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14. ABSTRACT

This project focused on the design and integration of biological sensors with nanoelectronic circuitry, using a diverse array of live redox proteins and biomolecules and based on the development of innovative linkers between the biological systems and the nanodevices. Six different redox proteins (GOx, ADH, GO, LAX, ChOx, Hb) were assembled onto carbon nanotube array electrodes in a site-specific manner by complementary DNA hybridization. The DNA links are renewable and show direct electron transfer. Engineered peptide linkers assembled Npx to nanostructured Au electrodes, providing sensitive detection of peroxide. A membrane-capture fabrication method integrated gold nanopore sensor elements with on-chip micro cyclic voltammetry (demonstrated with GOx). These results encompass (1) a diverse array of biomolecules (seven redox active proteins, plus DNA itself); (2) innovative linkers, including addressable links based on DNA and engineered synthetic biological links; and (3) multiple functional biological-to-nanoelectronic interfaces, including carbon nanotubes, gold nanowires, nanodots, and nanopores, both

15. SUBJECT TERMS

Biosensing, nanoelectronics, molecular linkers, biofunctionalization, carbon nanotubes, nanoelectrode ensembles, biomolecules, microfluidics, biodetection, redox proteins, self-assembly, bionanowires, photosynthetic reaction centers, nanodots, nanopores, electrochemical detection, engineered peptides

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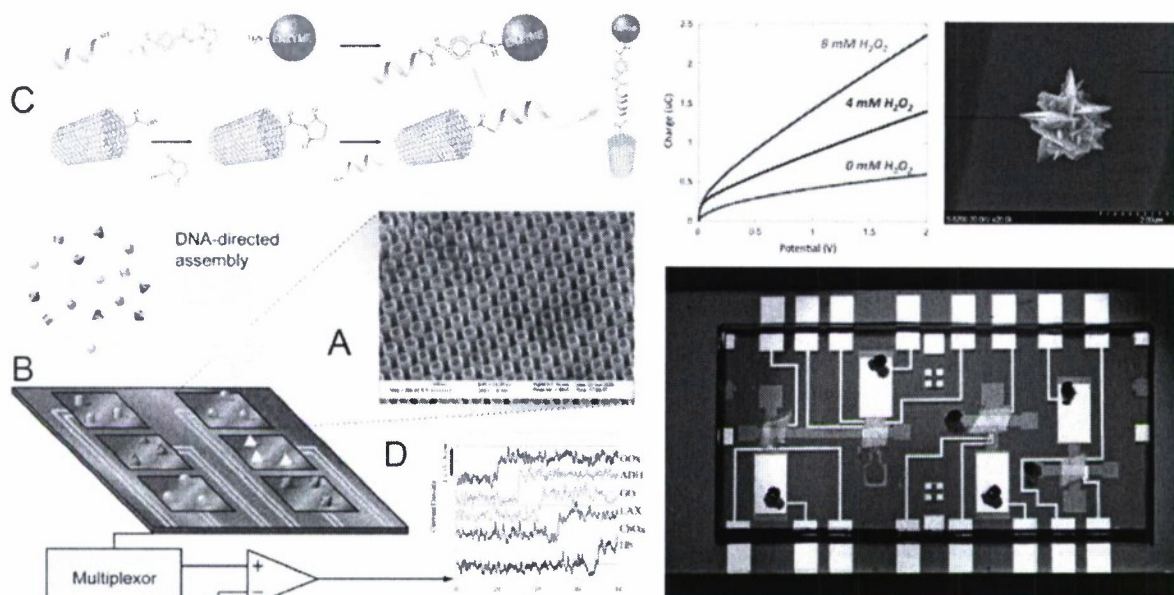
Direct nanoscale conversion of biomolecular signals into electronic information

MURI Final Report: 09/2003 – 05/2008

MURI themes and participants:

- **Molecular linkers** – interface between biological systems and electronic platforms: designs for efficient signal transduction; chemical linkage strategies, signal propagation
- **Biomolecule, Enzymatic systems** – source of specificity and signal generation: specific biomolecule systems; enhancing activity and reactivity lifetimes; targets of detection
- **Nanoelectronics** – physical properties; bio-functionalization; bio-electrical transduction and interaction
- **Nano&Micro Platforms**: integration of biological assemblies, electrodes, and microfluidic sensing arrays

Shana Kelley, University of Toronto (formerly at Boston College): DNA linkers and nanoelectrode ensemble detection

Joanne Yeh, University of Pittsburgh Medical Center: peptide linker systems, redox protein assemblies

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Nikolai Lebedev, Naval Research Laboratory and University of Virginia: photosynthesis protein assemblies

Irwin Chaiken, Drexel University: peptide linker systems

Jimmy Xu, Brown University: nanotube and nano-pillar arrays, fabrication, functionalization, site specific linking, bio-electronic transduction

Chuck Martin, University of Florida: nanopore platform for detection

Rod Beresford, Brown University: micro-fluidic chips and in-situ electrochemistry

The MURI team is pleased to report that it has accomplished all program goals it set out to accomplish at the start of the program, and has exceeded them on several fronts. In this final report, the MURI team members update their performance metrics to reflect the final 1 year of the program, and also compare the program accomplishments to the goals set forth in the original proposal.

MURI proposal specific goal: The design and integration of biological sensors with nanoelectronic circuitry, using a diverse array of live redox proteins and biomolecules and based on the development of innovative linkers between the biological systems and the nanodevices.

Specific goals accomplished are illustrated in the panels at the top of this report. Six different redox proteins (GOx, ADH, GO, LAX, ChOx, Hb) were assembled onto carbon nanotube array electrodes in a site-specific manner by complementary DNA hybridization. The DNA links are renewable and show direct electron transfer. Engineered peptide linkers assembled Npx to nanostructured Au electrodes, providing sensitive detection of peroxide. A membrane-capture fabrication method integrated gold nanopore sensor elements with on-chip micro cyclic voltammetry (demonstrated with GOx). These results encompass

- A diverse array of biomolecules (seven redox active proteins, plus DNA itself)
- Innovative linkers, including addressable links based on DNA and engineered synthetic biological links
- Multiple functional biological-to-nanoelectronic interfaces, including carbon nanotubes, gold nanowires, nanodots, and nanopores; both embedded in membranes and directly grown on chip; and integrated with planar microelectronic technology.

Thus, the specific proposal goal of direct electronic read-out from five redox proteins was met and exceeded.

MURI proposal long-term research goal: Scalable protein-specific self-assembly of protein-nanoelectrode arrays, and thus reliable and efficient bio-digital conversion interfaces.

Significant and pioneering contributions towards the long-term goals of bio-digital conversion are evidenced by the demonstration of protein-specific self-assembly of multiple proteins to an integrated electrode array, and their real-time sensory response and electrochemical functionality, including programmability, reconfigurability, and renewability. One may view the significance of this research initiative (MURI) in exploring and proving the feasibility of making

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bio-transducers and their integrated circuitry as a biological analog of the advent of semiconductor transistors and integrated circuits. Other important contributions include greatly enhanced electron transfer rates at nanostructured electrodes (from one to three *orders of magnitude* compared to bulk or micro electrodes) and on-chip precision calibrated reference electrode system for cyclic voltammetry at the nanoscale.

Thus, the long-term research goal was shown to be feasible, and enabling methods were demonstrated that facilitate future progress. These accomplishments show that the MURI goals were met as originally proposed, and that the findings from this program have the potential to revolutionize biosensing by leading a paradigm change in biosensing from detection of the presence of a particular substance to direct and real-time monitoring of the activities of targeted redox proteins. The DoD impact of this research program is that the direct electronic access to biomolecular reactions enables real-time detection of chemical and biological threat agents in the environment, plus real-time *in vivo* detection of human responses to toxins, pathogens, and other stressors. Thus, this research supports automatic surveillance and enhancement of the fitness and survivability of personnel.

The following table gives more explicit details on how the component sub-goals of the research proposal were met, and in most cases exceeded.

Proposal Goal	Major Achievements
Development of protein-DNA and protein-peptide linkers	1. We have developed different DNA linkers to assemble 5 different redox proteins that were proposed to serve as the demonstration vehicle for integration and scaling. 2. Six different redox proteins, exceeding the proposed objective, were assembled to CNT array electrodes in a site-specific manner by complementary DNA hybridization; the links are renewable, stable, and showed direct electron transfer. 3. Production and characterization of a library of peptide and PNA linker as bionanowires. 4. Design and synthesis of an innovative RC-specific peptide linker (Helix-37) for binding various inorganic and organic compounds to the protein
Enhancement of the conductivity of DNA and peptides	1. Site-specific assembly of GOx on CNT tips has yielded 1000× increase in electron transfer from enzymes to the electrode over that of conventional planar electrodes. 2. Metalized DNA has shown improved electron transfer efficiency. 3. Developed protein-electrode wiring using cytochrome C: improves electron transfer from photosynthetic reaction center by more than 100×.
Linking sensor molecules to nanoelectrodes	1. Redox enzymes were linked to sidewall or tip of CNT selectively via physical adsorption or chemical linking and the electron transfer activity was compared between two methods. 2. We developed a new approach to the biofunctional derivatization of carbon nanotube arrays, modifying the tips and sidewalls of CNTs using two different coupling chemistries. 3. We demonstrated that modular coiled coil heterodimers could function as linkers and could be assembled specifically on CNT tips. 4. Constructed 8 protein mutants for specific oriented binding of RC protein to electrodes 5. Integrated bionanowires with enzymes and electrode surfaces for formation of fully functional biosensors, capable of detecting toxicological and cellular markers.

Synthesis of nanoelectrodes and arrays	1. Highly ordered CNT nanoelectrode arrays were developed and routinely delivered to the collaborators in the team and beyond. 2. We investigated a new type of electrode platform for biomolecular sensing — gold electrode ensembles — and modified them with DNA for ultrasensitive biomolecular detection. 3. Constructed a novel type of high functional density nano-organized photo-active materials with photosynthetic RC proteins encapsulated inside carbon nanotubes in CNT array, with 5-fold gain in photoelectrical conversion.
Fabrication of flow-cell chips	1. Calibrated Ag/AgCl reference electrode system was integrated in a microfluidic platform and tested with several targets. 2. Au nanodots and nanopores were integrated with planar electrode technology, combining efforts of several co-PIs to show high sensitivity (picomolar) detection limits. 3. Membrane-capture integration method was demonstrated with Au nanopore electrode (functionalized with GOx).
System integration for information extraction	0. A long-term undertaking that addresses one of the most profound challenges in extracting information from the collected biomolecular activity data. It is expected to continue well beyond this first 5-year phase devoted to feasibility studies. 1. Feasibility of electrochemical array sensing using commercial read-out integrated circuits was shown in a prototype design for a chemical imaging array based on MURI sensor modules.

MURI final year accomplishments and impact: This MURI program pursues methods and technologies that enable real-time monitoring, sensing, control, and actuation of biomolecular reactions and conversion of the bio-signals into digital information. The team consisted of seven co-PIs from six institutions following a small reorganization as recommended by the review panel at the end of the first three-year period. The team took a modular approach to achieve the program objective through the development and demonstration of: (a) novel and scalable nanoelectronic technology platforms based on carbon nanotubes, metallic nanowires, nanodots, and nanopores, (b) bio-molecular transduction units based on engineered redox and photosynthetic proteins, (c) molecular linkers for scalable, addressable self-assembling of the targeted proteins to nanoelectrodes and for efficient electron transfer from the proteins to the designated nanoelectrode, (d) micro-flow chips and integration of the nanoelectrode arrays in the flow-cells with read-out circuitry of ultra-high sensitivity.

As a tangible measure of success, the team committed to meeting the challenge of demonstrating an integrated array chip of multiple protein-electronic transducer units, with at least 5 self-assembled individually addressed units, by the end of this MURI program. The technology platform is designed to be inherently scalable to many cells of different proteins, and with ultimate sensitivity and control at the level of hundreds of protein molecules in each cell. This goal has been successfully reached, and exceeded on several fronts.

This MURI program represents an unprecedented exploration of the feasibility of direct and real-time conversion of biomolecular signals into digital electronic information. As expected, it has met numerous and often unprecedented challenges. To meet these challenges, the team has worked on the development, test, selection, and assembling of a large number of building

blocks for the eventual and unprecedented integrated array of nano-biomolecular-electronic transduction devices that bridges the two vastly different worlds of biological systems and physical electronics systems.

In the past year, the program has continued a highly productive trend involving substantive collaboration among the multiple laboratories, especially in regards to functionalization of carbon nanotubes, protein specific immobilization, and linking to designated nanoelectrodes, real-time detection and monitoring of proteins and their activities, and characterization of highly sensitive target modules. Significant advances also have been made on the front of microfluidic flow-cell arrays, including integration of both nanowire and membrane nanopore sensing elements in a planar chip format, and hardware / software design for information extraction.

These accomplishments show that the MURI goals were met as originally proposed, and that the findings from this program have the potential to revolutionize biosensing by leading a paradigm change in biosensing from detection of the presence of a particular substance to direct and real-time monitoring of the activities of targeted redox proteins.

Performance metrics: The PI and co-PIs together are reporting the following status since the project start approximately 5 years ago (Sept. 2003):

- 122 journal publications appeared or in press, including contributions to *Science, Proc. Natl. Acad. Sci., IEEE Proc., Anal. Chem., J. Am. Chem. Soc., Nano Letters, Appl. Phys. Lett.*

- 93 conference papers presented, in both specialized and broader audience meetings, domestic and international

- 151 invited talks / consultations / technology transitions, including industry, academic, and government laboratory collaborations

- 13 patents, either awarded, filed, or in preparation

- 5 new course starts, and new content in 6 more courses

- 25 significant awards, including Guggenheim and Sloan Fellowships, Fellows of the Electrochemical Society and Institute of Physics, Best Ph.D Thesis in Engineering Award, Sigma Xi Prize, and high-profile innovator citations

Highly Efficient and Integrated Bio-Nanoelectronics Using Addressable and Programmable Molecular Linkers as Site-Specific Enzyme-Nanoelectrode Interfaces

Jimmy Xu

This section summarizes the major achievements of the group led by Professor Jimmy Xu at Brown University, including both in-house explorations as well as some of the collaborative efforts with other members of the MURI team. It follows the path of the significant milestones reached over the course of this project, beginning with the early work in interfacing redox proteins with our nanoelectronic platforms, identification of the most productive linking strategies, characterization of the electron transfer, the incorporation of different molecular linkers to direct enzyme assembly at the electrode surface, and using these linking strategies to enable the reversible assembly of multiple different enzymes to distinct nanoelectrode regions for the construction of scalable, renewable, multiplexed bio-nanoelectronic devices.

The GOx-CNT bio-nanoelectronic transducer. As a proof-of-concept study designed to observe redox enzyme-to-CNT electron transfer, we covalently modified a highly-ordered CNT array electrode with glucose oxidase using NHS/EDC carboxyl-amine coupling chemistry and placed it in a flow cell under a near-constant flow of buffer solution. After determining the baseline current at a constant potential of 0.4 V, a glucose solution was injected at timed intervals, and the resulting rise in current was measured (Figure 1). The current arose from the two-electron oxidation of glucose by the immobilized glucose oxidase. The system was also stable over a wide range of pH (4.0 – 9.0).
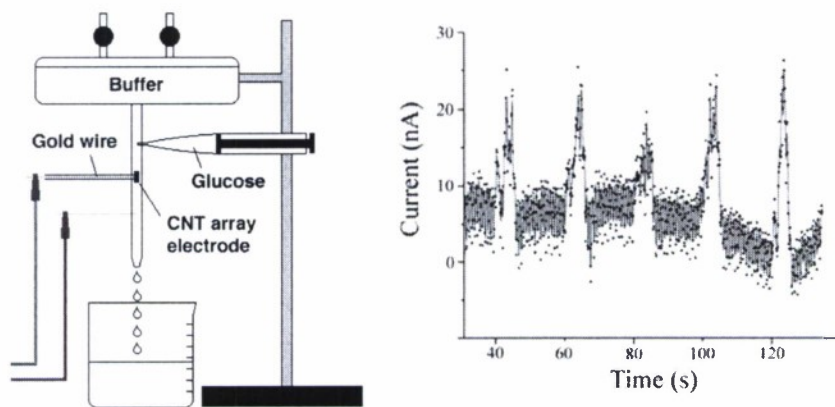
Figure 1.

On a similarly prepared CNT electrode, cyclic voltammetry (CV) was performed to further characterize the GOx-CNT conjugated system. A very distinct peak occurred around 0.4 V, which is the expected redox potential of GOx. Various glucose concentrations were used in the study, and a direct relation was found between concentration and CV response. This result gave initial promise to the application of this system as a protein biosensor.
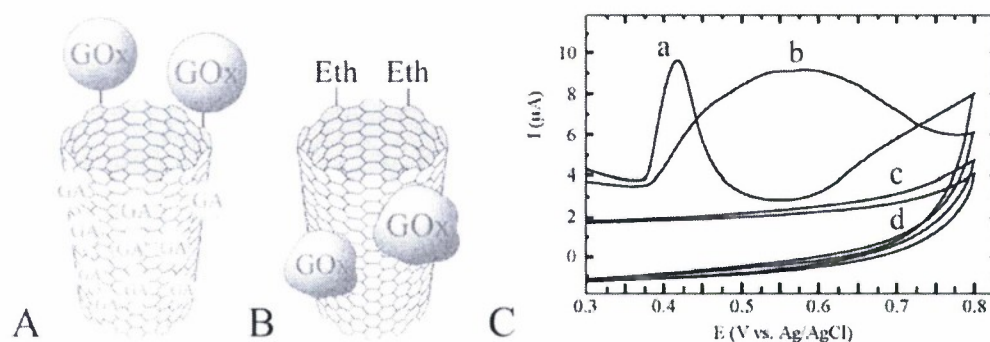
Figure 2.

Examination of site-selective linking strategies. The group then examined the properties of the protein-CNT conjugate with two different methods of protein immobilization. On one CNT electrode, glucose oxidase was covalently bound to the tips, and the hydrophobic adsorption to the side-wall was prevented by the use of a surfactant (A). On another electrode, the protein was allowed to adsorb to the walls of the tubes, but the tips were capped so that no covalent bond could be made (B). CV was then performed on the two electrodes (C). It was found that the protein linked to the tips gave a very sharp peak at 0.4 V (a), while the protein adsorbed to the side-walls gave a much broader peak (from 0.4 – 0.7 V, b). This, we expect, is due to the fact that the adsorbed protein exists over a range of somewhat denatured states, and is therefore less active than the protein linked to the tips, which is still in its active conformation. Furthermore, in terms of the amount of protein bound (based on available surface area to attach) the adsorbed protein outnumbers the covalently-linked protein by more than an order of magnitude, but the current response of each species is almost identical. This shows not only the importance of preserving the natural active conformation of the protein, but also the advantage of linking to the tips, where electron transfer is intrinsically more efficient than at the side-wall.

Highly enhanced electron transfer rate. Saturation and sensitivity assays were performed to determine key performance metrics, namely the k_{ET} and the detection limit of the system. We found our system to exhibit an astounding unimolecular k_{ET} of 1500 s^{-1}. This is a value that is nearly three orders of magnitude greater than adsorption-based systems currently in literature, and is comparable to optimized systems involving specialized molecular linkers used to directly access the redox center. Furthermore, this rate is more than twice the rate at which molecular oxygen, the natural electron acceptor for GOx, receives electrons from the enzyme *in vivo*. A detection limit in the nanomolar range was achieved, which compares very impressively to comparable systems that generally do not venture below the millimolar range.

Photosynthetic reaction center studies. Although glucose oxidase was commonly used as a model enzyme, we have explored the use of many different electroactive proteins in the course of this project. In collaboration with the Lebedev group, we succeeded in delineating the factors responsible for efficient biophotovoltaic responses of photosynthetic reaction centers (RC). Together we characterized and compared responses of RC contacted by carbon nanotube

(CNT) arrays and RC contacted by highly ordered pyrolytic graphic (HOPG) under potentiostatic conditions in a three electrode photoelectrochemical cell. RC was immobilized on the carbon substrates using a Ni(NTA)-pyrene self-assembled monolayer (SAM) for protein attachment to the basal plane of HOPG or the open end of the CNTs. With ubiquinone 10 (Q2) as the main electron acceptor in solution, RC on CNT arrays gave rise to faster photo-induced electron transfer kinetics and 5 times greater photocurrent density (at 0.05 V vs NHE with excitation > 700 nm at 0.05 mwatts/cm^2).
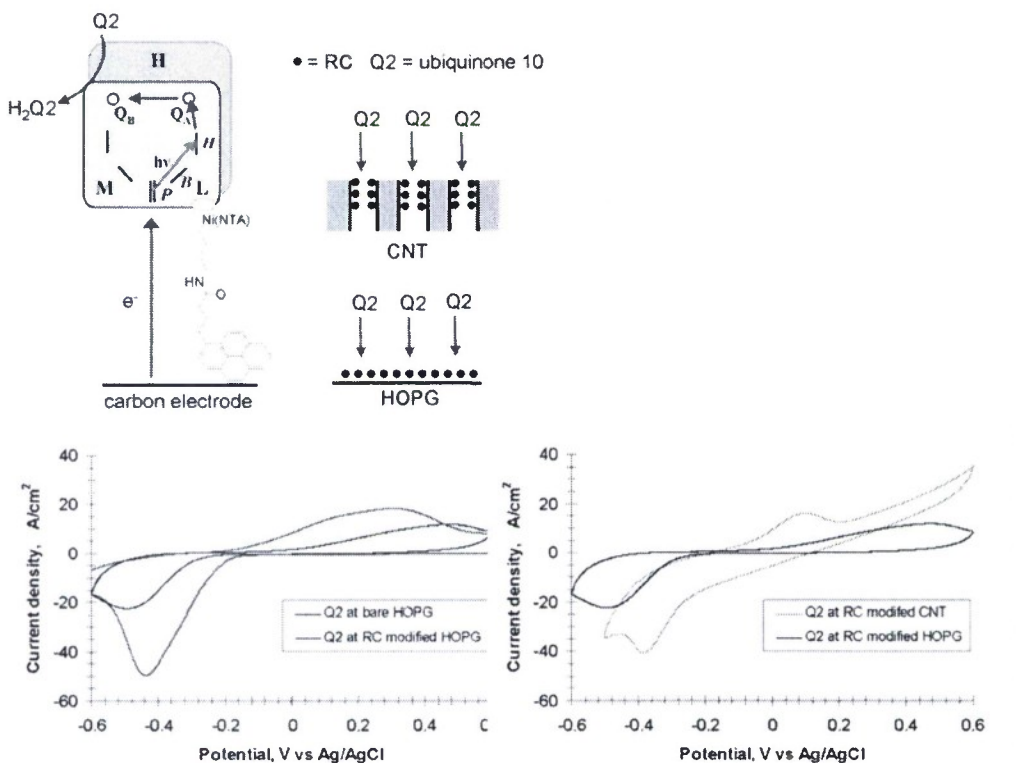
Figure 3.

In addition, fluorescence analysis of pigments extracted from the surfaces showed a higher surface density of RC per unit area of the CNT arrays (27 ± 5 x 10^{-12} moles/cm^2) compared to the RC immobilized on a HOPG substrate (7 ± 1 x 10^{-12}moles/cm^2). Interestingly, under illumination of high light intensity, RC on CNT arrays displayed a unique kinetic photoresponse suggesting that the concentration of the electron acceptor, Q2, in the pore structures of the CNTs was being rapidly depleted. This kinetic phenomenon was not observed for RC modified HOPG.

Site-specific linking of nanostructures by metallizable peptides. In collaboration with the Yeh group, we demonstrated controlled and confomationally defined linking of redox proteins to carbon nanotube electrodes with the use of designed electron conduits made of engineered peptides which act as bio-electrode as shown below. The engineered peptide bioelectrode is

formed through the coordination of an Au nanoparticle (AuNP), cobalt-metallized peptide, and the enzyme NADH peroxidase (Npx).

With the use of the bioelectrode, redox enzyme activities in response to a specific ligand or other physiological signal can be wired to and read out electronically by a nanoelectronic circuitry. Utilizing the NADH peroxidase system, enzyme based assemblies are coordinated to electrodes, including CNT nanoelectrode arrays, that sense reactive oxygen species such as hydrogen peroxide and send a detectable electrochemical signal. SEM analysis confirms that the various linking strategies allow specific linkage of enzyme-metallized-peptide bio-assemblies on the tips of CNT electrodes with various levels of incorporation, as designed.

Electrochemical analysis further indicates that the signal is readily detected with high sensitivity and the amounts of substrates can be quantitated by this approach. This collaborative work has allowed us to integrate the various results towards building highly sensitive real-time biosensors on a nanoplatform capable of detecting substrates with extreme sensitivity.

Figure 4.

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CNT22NHS
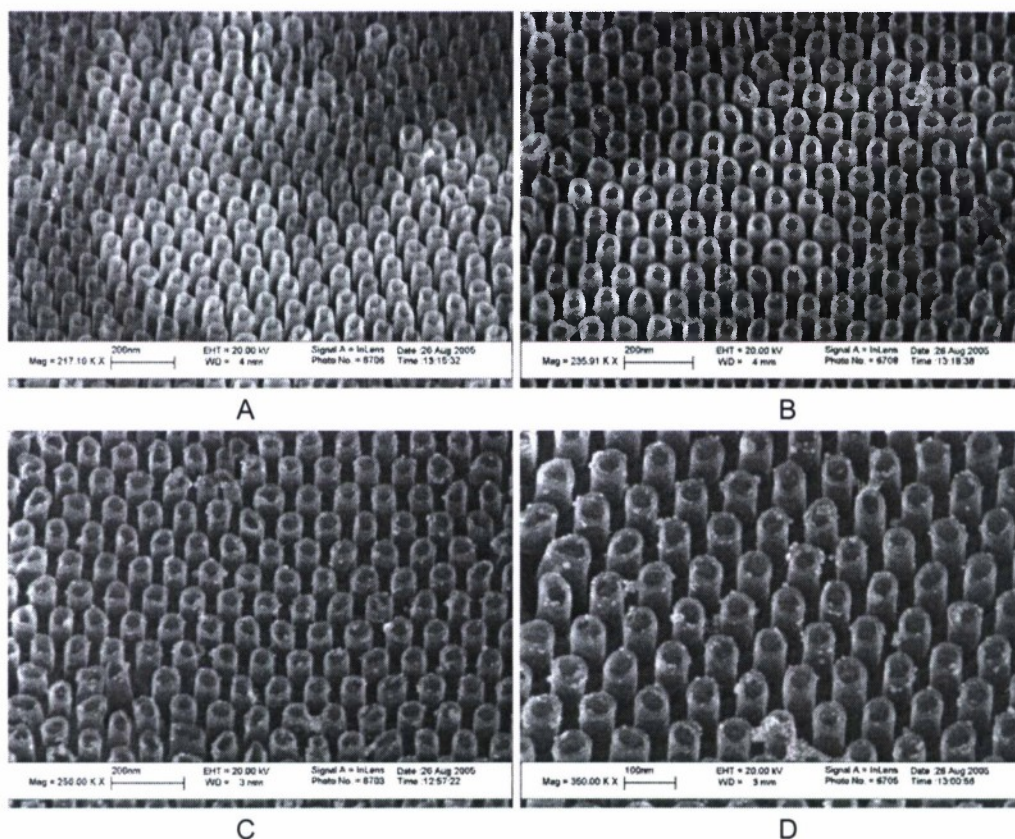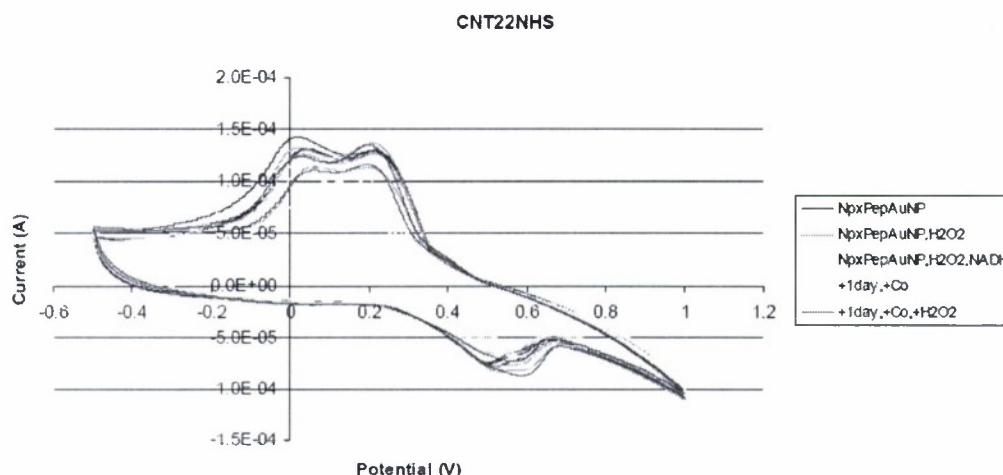
Figure 5. Cyclic Voltammetry of Npx-based Bio-assembly. Superposition of CVs of enzyme bio-assembly on Au-wire (top) and CNT electrodes (bottom). Ag/AgCl reference electrode was used for the CNT electrode measurements while the Au-wire was used a pseudo-reference electrode for the Au-wire bio-assembly measurements. As suggested by these results, the detection levels using the CNT electrodes is at least an order of magnitude greater with the CNT electrodes, a phenomenon under active study.

DNA-directed self-assemblying of catalysts onto carbon-nanotube and growth of semiconductor-carbon nanotube heterojunctions. We have harnessed the extraordinary specificity of DNA to site specifically place gold catalyst nanoparticles for the generation of co-axial multi-material nanowires. This work along with the site specific targeting of multiple DNA strands to a highly ordered CNT array took place in close collaboration with the Kelley group and has resulted in three manuscripts. We have also had success in interfacing coiled-coils with the Chaikin group exploiting the various chemical functionalities of the dual peptide system. Our system relies on the site-specific attachment of a DNA oligonucleotide to arrayed nanostructures. The subsequent delivery of a catalyst through hybridization of a complementary sequence provides a platform for the growth of a second type of nanowire, and is an inherently parallel and scalable process thus addressing a main challenge of multi-material nanowire fabrication. This approach is

Figure 6. Site-specific delivery of DNA oligo-nucleotides and appended Au nanoparticles. SEM images shown in (A) and (B) correspond to nanoparticles introduced through parallel hybridization following the serial attachment of two DNA single strands. Scale bar is 50nm.
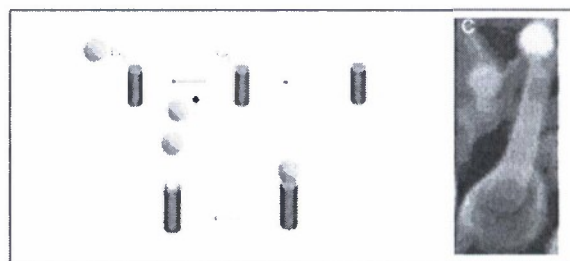
Figure 7. Hybrid nanostructure design templated with DNA assembled catalyst.

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the first to generate a one dimensional, optically active, multi-material nanowire platform using site specific catalyst placement via biological self-assembly.

Integration of a DNA molecular linker. The next step was to incorporate a universal, addressable molecular link to assemble proteins to the nanoelectrode. This self-assembling strategy would allow us to wire any number of proteins to the appropriate docking sites, addressable by the molecular link. DNA oligonucleotides were the clear choice to investigate, given their high specificity, addressability, strength, and controllable reversibility of binding. At first, gold nanoparticles were considered for use as an intermediate linker, since they would simplify visualization and qua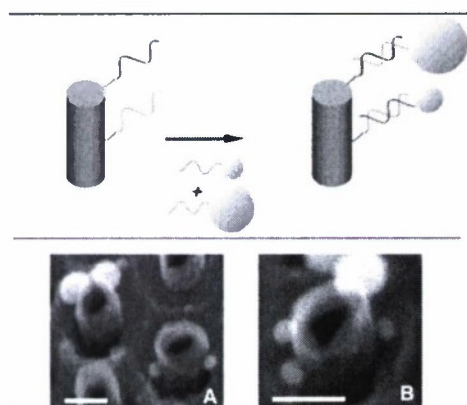ntification procedures. After many failed attempts at achieving the simultaneous binding of proteins and thiolated oligos to the same nanoparticles in the desired stoichiometry, it was decided that a more direct linking scheme was preferable. N-succinimidyl[4-iodoacetyl]aminobenzoate (SIAB, Figure 8) was chosen as a linker to conjugate the enzyme and DNA oligos. The heterobifunctional nature of SIAB ensures that one side will bind a protein and the other will bind a thiolated DNA oligo. The NHS ester of SIAB is reactive towards amine groups, which are prevalent on all proteins in the form of lysine, arginine, asparagine and glutamine residues, as well as the N-terminus. The iodoacetyl group will react with the thiol functional group of a synthetic oligo. To test the ability to achieve enzyme docking on the CNT array by hybridization of complimentary oligos, a CNT array electrode was covalently modified with amine-terminated oligo1 (o1). The electrochemical signature of the o1-linked array was then measured by CV (Figure 9a). Next, an oligo complimentary to o1 (o1') was conjugated with GOx. The GOx-o1' conjugate was then introduced to the array, incubated to allow hybridization, and then washed to remove any adsorbed protein. CV measurements were then performed to test for DNA-linked GOx activity (Figure 9b). As the figure shows, a unique set of oxidation and reduction peaks appear upon the hybridization of GOx to the array (marked by arrows). The peaks are glucose concentration-dependent.
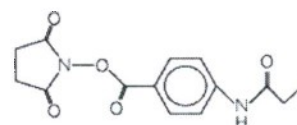
Figure 8. Structure of the heterobifunctional linker, SIAB.
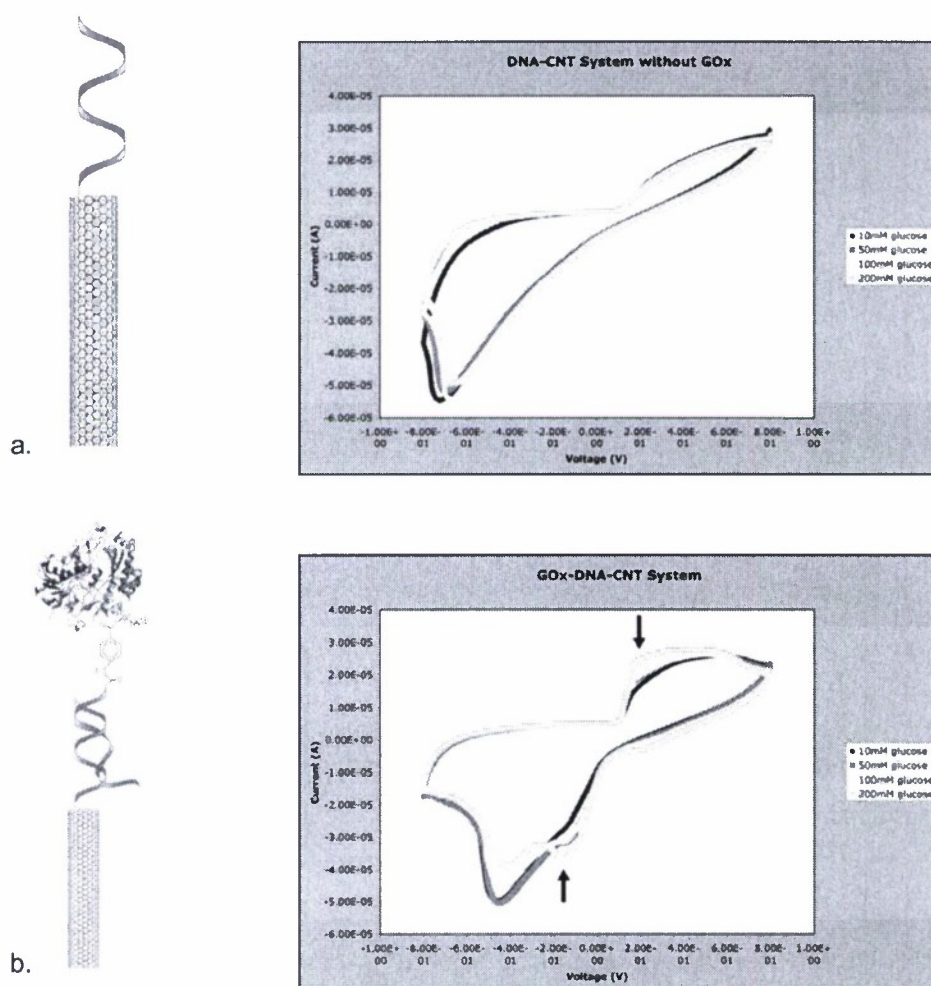
Figure 9. Cyclic voltograms of the o1-linked CNT array electrode before (a) and after (b) hybridization with o1'-GOx conjugates.

This fulfills the A-B-C objective of the project, recording a meaningful biological signal from a protein, through a self-assembling molecular link (DNA) to a nanoelectrode interface (the CNT array). To our knowledge, it is the first such recording of a biological event through an addressable, self-assembling molecular link. What is more exciting is that this linking mechanism is, in principle, interchangeable for all proteins, since it relies only on the presence of an amine functionality, of which all proteins have at least one, and most have many. Furthermore, by selecting oligos with different functionalities, this strategy is transferable all commonly-used electrode types (gold, carbon-based, etc.). It can therefore be used on any nanoelectronic platform that we choose.

Prevention of non-specific protein adsorption. An important consideration in the design of an addressable assembly scheme is eliminating any alternative, non-site-specific means of binding. A molecular linker designed to deliver its payload to a specific, pre-programmed binding region will be of little value if, at the same time, the payload is attaching itself to random sites by

some alternative immobilization. On the DNA-modified CNT array, there are two avenues through which ssDNA-conjugated enzymes can attach to the surface. The first and preferred method is through sequence-specific hybridization of complementary ssDNA tags, which will guide the enzyme to its intended position. The second means is by hydrophobic adsorption to CNT side walls, which can occur anywhere on the CNT array. Although this adsorbed enzyme will likely exhibit relatively low bioelectrocatalytic activity due to the denaturing interaction with the hydrophobic surface, in sufficient quantities it can still potentially contribute a significant amount of "noise" to the overall system. Figure 10A illustrates the case in which no preventative measures are taken to avoid adsorption of enzyme. Each enzyme is hybridized to its designated CNT by the DNA link, but at the same time, both enzymes are adsorbed to each tube. In this case, the electrochemical signature of each enzyme will be observed from the two binding regions, and de-convolution of the true signal from the interfering signal can be exceedingly difficult.
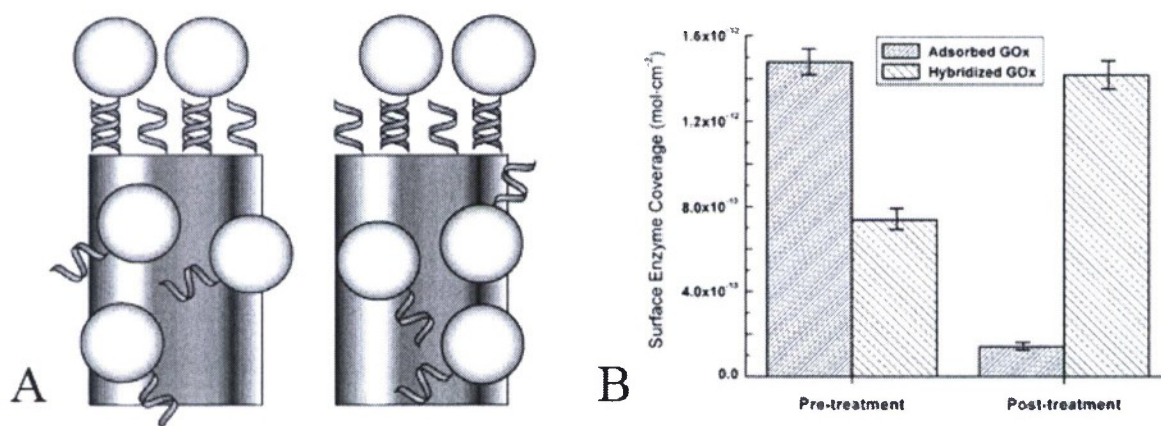
Figure 10. Controlling enzyme adsorption to CNT side walls. (A) Without the use of surfactants, protein will adsorb randomly to the side walls of CNTs. These adsorbed enzymes will produce background noise that clouds the signal from enzyme that is immobilized through intentional, DNA-directed linking. (B) The amount of active enzyme bound to the array by DNA hybridization and adsorption, both before and after surfactant treatment of CNTs. Through the use of surfactants, the ratio of hybridized to adsorbed enzyme can be increased twenty-fold.

It has been shown that surfactant treatment can be very effective in preventing adsorption of protein to the side walls of CNTs. Such a coating can therefore be used to ensure that the primary means of enzyme attachment is by DNA-directed immobilization. To test this strategy, a non-complementary ssDNA-GOx conjugate was first allowed to adsorb extensively to ssDNA-modified CNTs. Using CV, the surface coverage of the adsorbed enzyme was determined to be $1.5\pm0.1 \times 10^{-12}$ mol·cm^{-2} by integrating the anodic current peak to find the total charge associated with the FAD redox units. After the addition of a complementary ssDNA-GOx conjugate and subsequent hybridization of protein to the CNT tips, a surface coverage increase of $7.6\pm0.1 \times 10^{-13}$ mol·cm^{-2} was observed. This same experiment was repeated, this time after treating a ssDNA-modified CNT array with GA to resist protein adsorption. After surfactant treatment, the

quantities of adsorbed and hybridized protein were found to be $1.5\pm0.1 \times10^{-13}$ mol·cm^{-2} and $1.4\pm0.1 \times10^{-12}$ mol·cm^{-2}, respectively. These quantities are presented in Figure 10B.

In short, the surfactant treatment boosts the quantity of hybridized protein approximately twenty times relative to the quantity of adsorbed protein. The ratio of hybridized enzyme to adsorbed enzyme, which will dictate the signal-to-noise ratio in our multi-functional biosensor, improves from 1:2 to 10:1. In other words, before treatment with surfactant, the signal from hybridized enzyme is clouded by the errant electrochemical signature of randomly adsorbed protein. After surfactant coating of the CNTs, this adsorption does not occur and the signal from site-specifically addressed and bound protein is clearly distinguishable. The near two-fold increase in hybridization efficiency following surfactant treatment is also an interesting observation that may be attributed to reduced steric hindrance in the absence of adsorbed GOx. By eliminating protein adsorption and freeing up surface area, there is more opportunity for DNA-tagged enzyme to bind.

The nanoneedle platform. We have developed a new CNT electrode platform termed a "nanoneedle" that, when functionalized with GOx, is capable of low-limit detection of glucose. Furthermore, the nanoneedle is able to perform measurements in very small volumes of solution, which will enable substance detection even in cases where sample volume is extremely limited.
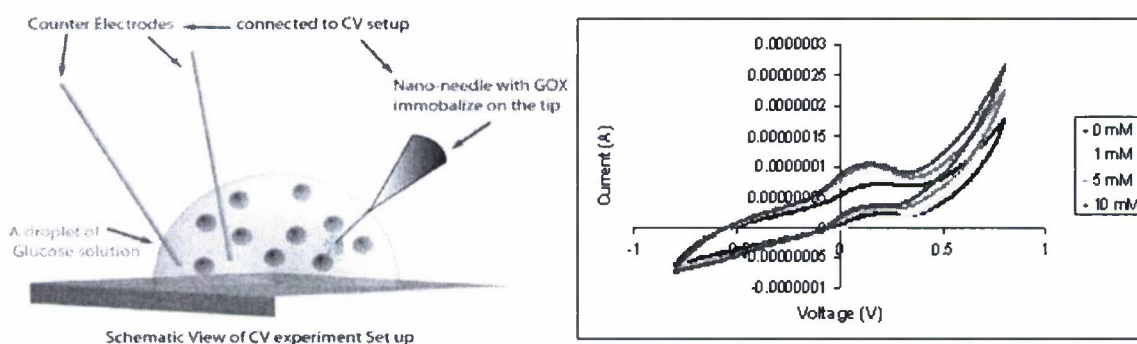
Figure 11. The CNT nanoneedle electrode for glucose detection. (Left) A schematic drawing of the nanoneedle functionalized with GOx, inserted into a small volume of glucose solution. (Right) Resulting CV measurements performed successively under increasing glucose concentration. The increase in glucose concentration is marked by an increase in peak current response.

Wiring efficiency. A great amount of effort was invested in determining the wiring efficiency of our DNA linking system described above. To summarize the linker design, a thiolated single-stranded DNA oligo (ssDNA) is cross-linked to a redox enzyme using the cross-linking agent N-succinimidyl[4-iodoacetyl]aminobenzoate (SIAB). Our CNT array electrode is functionalized with the complementary ssDNA by NHS/EDC carboxyl-amine coupling. Hybridization of the two strands results in the physical tethering of the redox enzyme in close proximity to the CNT tips, and we have demonstrated a direct transfer of electrons across this molecular linker. There

remained an important question, however, of the efficacy of this linker in establishing an efficient electrical contact to the underlying electrode. Specifically, we intended to quantify (1) the degree to which enzymes remained catalytically active following these cross-linking and hybridization procedures and (2) the amount of enzyme of the total population of bound enzyme that establishes an efficient electrical contact to the CNTs. To do this, three independent assays were performed using glucose oxidase (GOx) as a model enzyme, pictured in Figure 12. First, the amount of active GOx that is electrically contacted to the array was determined by taking CV measurements in the absence of glucose and integrating the oxidation peak from that arises after hybridization of GOx-ssDNA conjugate to the electrode (Figure 12, (1)). The total number of electrons transferred can be calculated and this value can be correlated to the number of successfully "wired" GOx. By this method, we found the average surface enzyme coverage (SEC) of GOx successfully electrically contacted to the CNTs to be $1.3 \pm 0.06 \times 10^{-12}$ mol/cm^2. The second characterization quantified the total amount of active protein (both contacted and not contacted to the CNTs), using an Amplex® Red Glucose/Glucose Oxidase Assay Kit from Molecular Probes. In brief, the oxidation of glucose is coupled with the conversion of 10-acetyl-3,7-dihydroxyphenoxazine (the Amplex® Red reagent) to the red-fluorescent oxidation product resorufin (Figure 12, (2)). By monitoring the rate of formation of resorufin by fluorescence spectroscopy and comparing to standard samples, we can determine the amount of active GOx present. The average SEC of total active GOx was found to be $1.7 \pm 0.05 \times 10^{-12}$ mol/cm^2.
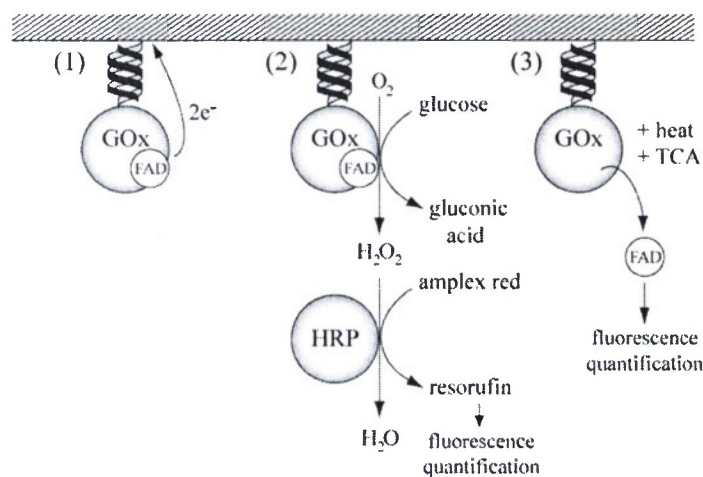
Figure 12.

Finally, to determine the total amount of GOx (both active and inactive) that is bound to the CNTs, we first removed all enzyme from the array by sonication for 2 hours at 65 °C in DIH$_2$0, which is sufficient to dehybridize the short oligo duplex. We then extracted the flavin molecules from GOx using a heat treatment and 5% w/v TCA, followed by centrifugation of precipitated apo-enzyme, leaving FAD in the supernatant (Figure 12, (3)). FAD concentrations can measured by fluorescence spectroscopy, and since each subunit of GOx contains a single FAD redox center, the total amount of FAD present is equal to the amount of GOx bound to the array.

After neutralization of the acidic solution with K_2HPO_4, fluorescent measurements were taken and compared against standard FAD solutions, indicating a total GOx average SEC of 1.9 ± 0.06 × 10^{-12} mol/cm^2. By correlating these values, a few significant conclusions can be drawn. Relating the amount of total active protein (2) to the amount of total protein (3), we see that 89±3% of all protein that is physically linked to the CNT array by this DNA linking scheme retains its bioelectrocatalytic function. Furthermore, correlation of the amount of active, contacted GOx (1) to the amount of total enzyme (3) reveals that 68±3% of the total protein has established an efficient electrical contact to the CNTs. Dividing the quantity of active, contacted GOx (1) by the amount of total active enzyme (2) tells us that 76±3% of all active GOx establishes an electrical contact to the CNTs. The DNA linking scheme designed here results in random enzyme orientation, and we can deduce that three-fourths of all binding orientations result in an efficient electrical contact.

Orientation control. Another question we sought to answer was whether the orientation of the enzymes could be controlled using our molecular linking scheme. In collaboration with the Kelley group, we performed a study in which the ssDNA tagged was bound specifically to the active site of the enzyme, and hybridization of this conjugate to complementary ssDNA on a gold nanowire electrode ensemble (NEE) resulted in the orientation of the active site toward the electrode surface. In this experiment, the single free cysteine group featured in the GOx structure was exploited to demonstrate that orientation control is possible using a variation on the SIAB cross-linking scheme described previously. Essentially, by reversing the binding chemistry as shown in Figure 13A, reacting the iodoacetyl group of SIAB with the single thiol on the enzyme surface and the succinimidyl ester with an amine-terminated ssDNA oligo, hybridization of this new conjugate with an ssDNA-modified electrode surface results in a single binding orientation for all proteins. Favorably, the one free thiol on GOx is located in the vicinity of the active site of the enzyme, and so this assembly scheme will specifically orient the active site towards the electrode surface. This was performed using a gold NEE as the electrode surface. The NEE was modified with thiolated ssDNA address strands.

Figure 13.

Three different assays were run: first with GOx-ssDNA conjugates featuring a sequence complementary to the address strand, then with a non-complementary conjugate and finally with unmodified GOx. The latter two trials were controls designed to demonstrate the sequence-specificity of binding and the advantage of 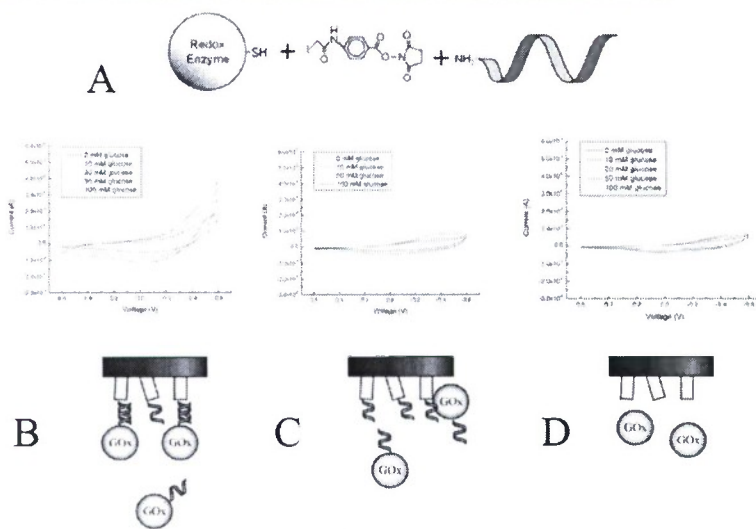hybridization versus adsorption, respectively. Figure 13 shows the results of these trials. It can be clearly seen that the complementary conjugate (B) shows a clear bioelectrocatalytic response to increasing glucose concentrations. Each of the controls (C,D) shows virtually no response to glucose injections. This indicates that assembly via the DNA linker is highly sequence-specific, and that hybridization is the primary method of enzyme attachment while protein adsorption plays an insignificant role. Although further characterization is necessary to quantify the relative performance of the optimally-oriented GOx assembly as compared to the randomly-oriented system, this study serves as a proof-of-concept that this oriented system is feasible and fully-functional for an enzyme that features a free thiol group, or that can be engineered to incorporate a thiol onto its outer shell.

Multiplexing. The major motivation behind the incorporation of a programmable molecular linker such as DNA is to direct the parallel assembly of multiple different enzymes for the creation of a multiplexed bioelectronic device. We have previously demonstrated the use of our DNA linking system to coordinate the assembly of two different redox enzymes, GOx and alcohol dehydrogenase (ADH) to two distinct sensing regions to create a multiplexed biosensor for the parallel detection of glucose and ethanol. In this phase of the project, we have extended the degree of multiplicity from two enzymes to six. GOx, ADH, galactose oxidase (GO), lactic acid oxidase (LAX), cholesterol oxidase (ChOx) and hemoglobin (Hb) were each tagged with a ssDNA oligo of a different sequence. These conjugates were combined in a common hybridization bath and allowed to assemble to their respective CNT array regions directed by sequence-specific hybridization to the complementary "address" strands, illustrated in Figure 14B. The resulting system constitutes a multiplexed biosensor capable of monitoring levels of glucose, ethanol, galactose, lactic acid, cholesterol and nitrous oxide in real-time.

To verify this, the electrode was placed into an electrochemical cell and chronoamperometric (CA) measurements were taken with the corresponding redox potential applied to each CNT region. At five-second intervals, each substrate was injected into the electrochemical cell in sequence, resulting in current steps from the various sensing regions. These responses are shown in Figure 14D. Importantly, each sensing region responds only to the injection of the substrate of the enzyme for which it is addressed. The fidelity of electrocatalytic response of each region to injections of its proper substrate proves the efficacy of DNA-addressability, as well as the specificity of each enzyme toward its respective substrate. The only significant exception is the CNT region functionalized with GO, which catalyzes the oxidation of not only galactose, but of all primary alcohols. As such, the GO-functionalized region responds to both ethanol and galactose injections. To give further quantitative evidence as to the broad applicability of our enzyme-ssDNA crosslinking scheme using SIAB, we first performed an electrochemical characterization of each individual enzyme after hybridization to the CNT nanoelectrode to determine such key figures of merit as electron transfer rates (k_{ET}'s) and substrate sensitivities. The results of these characterizations are presented in Table 1. As the

table shows, all enzymes in this study exhibit efficient electron transfer kinetics and high levels of sensitivity over a broad dynamic range, indicating high levels of bioelectrocatalytic activity and efficient electrical contacts.
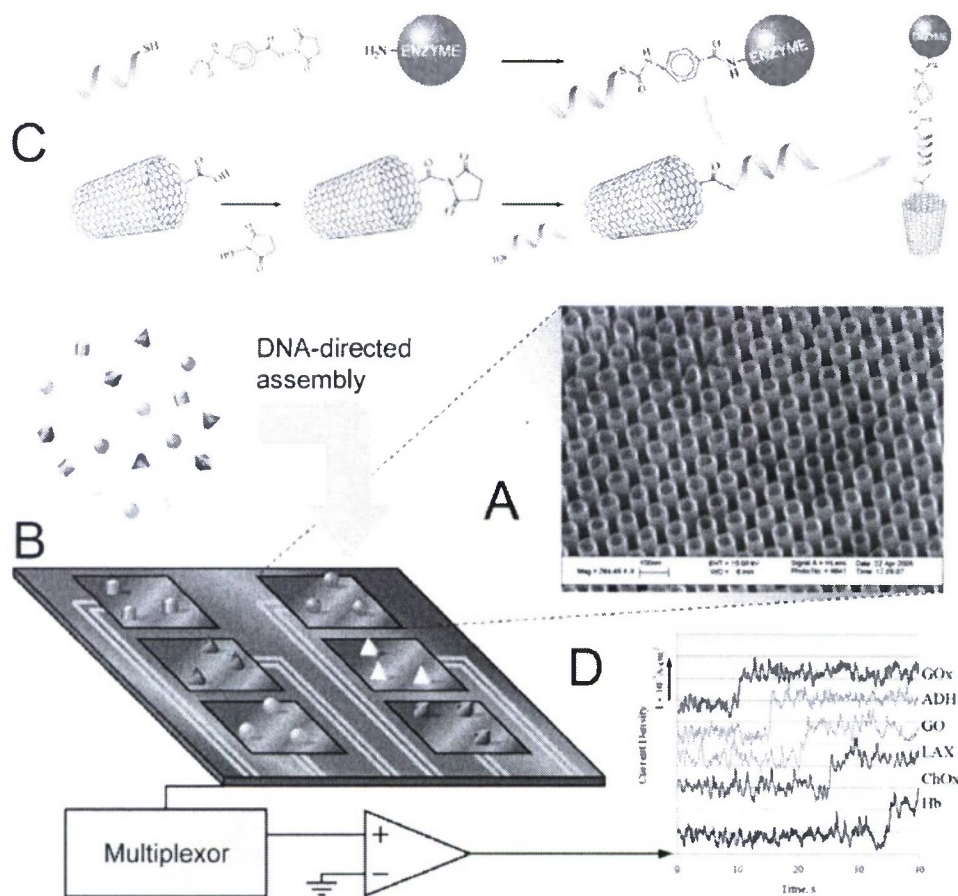
Figure 14. (A) Scanning electron micrograph of the highly ordered CNT electrode. The electrode surface is comprised of a hexagonal array of vertically-aligned CNTs grown from an anodized aluminum oxide nanopore template. (B) The DNA linking scheme is used to automate the site-specific assembly of different redox enzymes to distinct nanoelectrode regions on the sensor chip. (C) Enzyme-DNA cross-linking is achieved using SIAB. The complementary amine-terminated oligo is covalently bound to the CNT via an NHS-activated carboxylic acid group. Hybridization of complementary strands tethers the enzyme to the nanoelectronic platform. (D) CA detection of five different substrate injections in real time using five enzymes self-assembled to separate CNT electrode regions by sequence-specific hybridization. Injections of 1 mM glucose, 0.5 mM ethanol, 1.2 mM galactose, 0.2 mM lactic acid, 1 mM cholesterol, and 0.1 mM NO occurred at 10, 15, 20, 25, 30, and 35 seconds, respectively. These injection concentrations were chosen to induce comparable current steps. CA measurements of electrode regions addressed to bind GOx, ADH, GO, LAX, ChOx, and Hb are labeled accordingly.

Table 1. Characterizations of Five Redox-Active Enzymes Linked by DNA Hybridization

Enzyme	Approx. Linear Response Range ($\times 10^{-3}$ M)	Sensitivity ($\times 10^{-4}$ A/M/cm^2)	Active SEC ($\times 10^{-12}$ mol/cm^2)	Peak Current Density ($\times 10^{-6}$ A/cm^2)	k_{ET}
GOx	0 - 12	0.87	1.30 ± 0.1	1.72 ± 0.2	13.8 ± 1.4 sec^{-1}
ADH	0 - 50	1.0	4.96 ± 0.4	10.7 ± 0.8	22.4 ± 1.9 sec^{-1}
GO	0 - 10	0.77	1.92 ± 0.2	1.48 ± 0.1	8.0 ± 0.7 sec^{-1}
LAX	0 - 5	4.2	2.67 ± 0.3	2.90 ± 0.2	11.2 ± 1.0 sec^{-1}
Hb	0 - 2	8.9	3.78 ± 0.4	1.78 ± 0.1	4.9 ± 0.4 sec^{-1}

Based on CV measurements in 25 mM PBS, pH 7.0 at a scan rate of 50 mV·sec^{-1}. For the five enzymes GOx, ADH, GO, LAX and Hb the substrates glucose, ethanol, galactose, lactic acid and nitrous oxide were used, respectively. Quoted uncertainties are based on measurements of five separate trials.

DNA linker metallization. One further motivaton for selecting DNA as the molecular linker is its potential conductivity. It has been reported that native DNA can be converted to a more conductive form by incorporating divalent metal cations under high-pH conditions. For the purposes of our study, we seek to observe the effects of this conductivity increase on improving the performance of DNA-assembled bioelectronics and show that the metalization process does not destroy enzymatic activity. To characterize the metalized link, GOx was used again as the model enzyme, since it is fairly stable at high pH levels. The standard assembly was performed to hybridize the enzyme to the tips of a CNT array electrode, this time in a tris-buffered pH 9.0 solution and in the presence of Zn^{2+} ions. To try to distinguish the effect of metallization from that of simply increasing ionic strength by introducing metal cations, we have used Mg^{2+}, a non-M-DNA forming metal cation as a control. Additionally, a control was studied in which no metal cation was added. In each case the active, contacted SEC was determined electrochemically as described previously and correlated to the peak current density observed to calculate the different system k_{ET} 's. These values are tabulated in Table 2.

Table 2. Characterization of the Metallized DNA Linker using GOx

Linker Conditions	Active SEC ($\times 10^{-12}$ mol/cm^2)	Peak Current Density ($\times 10^{-6}$ A/cm^2)	k_{ET}	Relative k_{ET}
DNA	1.27 ± 0.1	0.69 ± 0.04	5.6 ± 0.4 sec^{-1}	100%
DNA+Zn^{2+}	1.37 ± 0.1	1.21 ± 0.1	9.2 ± 0.7 sec^{-1}	164%
DNA+Mg^{2+}	1.30 ± 0.1	0.82 ± 0.04	6.5 ± 0.4 sec^{-1}	116%

Based on CV measurements in 10 mM, pH 9.0 Tris-HCl buffer and 50 mM NaCl at a scan rate of 50 mV·sec^{-1}. Zn^{2+} and Mg^{2+} were added to final concentrations of 1 mM when used. 50 mM glucose was used as the substrate. Quoted uncertainties are based on measurements of five separate trials.

It is evident that these rates are lower than that observed at pH 7.0. This is the result of decreased bioelectrocatalytic activity in high pH solution. Regardless, there is a 64% increase in k_{ET} via the M-DNA linker compared to native DNA at this elevated pH level. The Mg^{2+} control

suggests that only 16% of this increase in electron transfer efficiency is the result of the divalent metal cations in solution. To further illustrate the effect of DNA linker metalization on improving enzyme-CNT electron relay, CV measurements were taken on the GOx-DNA-CNT system in the case of DNA, DNA + Zn^{2+} and a DNA + Mg^{2+} control. Figure 15A shows the peak current densities measured from each of the three systems in saturating glucose concentrations as pH is increased from 7.0 to 9.0. A pronounced increase in peak current is observed in the presence of 1 mM $ZnCl_2$ above pH 8.6, the established M-DNA conversion threshold. This current increase is superimposed on a decaying background that results from decreasing GOx activity in increasing pH. No peak current increase is observed in the case of the native DNA linker or in the presence of the divalent metal cation control. Finally, chronoamperometric measurements were taken for the three linker conditions at pH 9.0. After ten seconds, glucose was injected to a final concentration of 50 mM, resulting in current density steps from each of the three systems. These measurements are overlaid in Figure 15B. The relative magnitudes of the current density steps indicate increased linker conductivity in the case of the metalized DNA link. The increased electron transfer efficiency that is evident from these electrochemical characterizations of the metalized DNA linker suggest that it could play an important role in bioelectronics applications, serving as an addressable, reversible, conductive self-assembling junction. It should be noted that not all enzymes function well under elevated pH conditions. This limits the application of M-DNA to systems involving a subset of redox enzymes that are compatible with metalization conditions. Beyond bioelectronics, the DNA linker could also serve as a junction for more robust nanoelectronic devices whose elements (such as CNTs and metal nanoparticles) are less sensitive to environmental conditions.
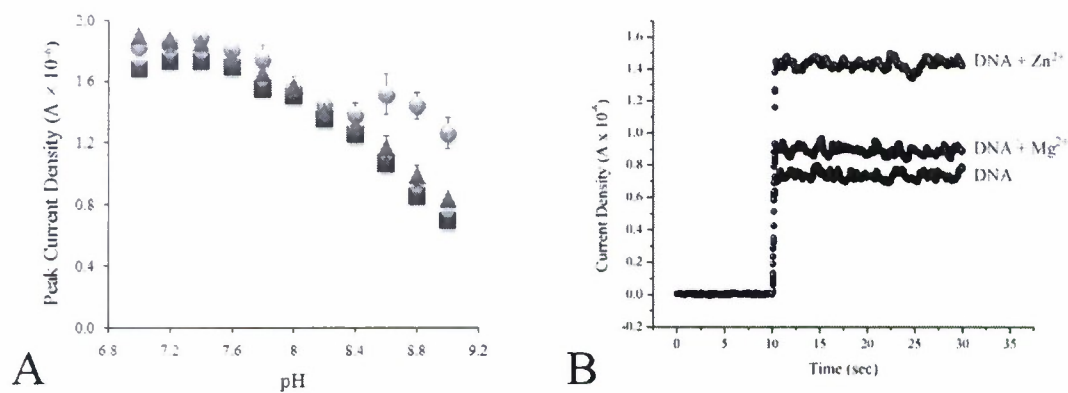

Figure 15.

Coordinated Biosensing:
Application of Three-dimensional Structural Information for Ultrasensitive Detection

Joanne I. Yeh

SUMMARY

The immediate goal of this MURI proposal was to design and integrate biological sensors with nanoelectronic circuitry, using a diverse array of biomolecules. To achieve this objective, innovative linkers and approaches for producing bio-based detectors ('bioassemblies') organized the biological components with the nanodevices in an optimal manner that maintained the activity of the biological materials while permitting integration with flow-cell or other platforms. The Yeh lab focused on the rational design, synthesis, modification, characterization, integration of various bio-based linkers, including peptides and nucleic acids and their incorporation with biological detectors, such as redox enzymes and protein receptors. As reported in earlier progress reports and published results, we have developed peptides capable of binding various metals with high affinities and targeted stoichiometries. In addition to peptide linkers, we designed and characterized nucleic acid-based linkers, including metalized DNA and peptide nucleic acids (PNAs). These linkers are conduits of the signals generated by the binding event of specific ligands or molecules; as such, these linkers are critical in transducing the signals from the biological detector to the readout device, such as an electrode. Our unique scientific perspective for these studies is in the application of three-dimensional atomic resolution information of the linkers and enzymes. As structural biologists, we specialize in the three-dimensional information of the linkers and proteins that provides a means of rationally targeting the analyte to be detected as well as precisely organizing the biological assemblies (ie the detection system) to maintain the functionality of the biomaterials while enhancing signal transduction events, such as electron transfer mechanisms. We call this approach of utilizing atomic resolution structural information for sensor development "coordinated biosensing" and have shown that the biosensors produced are robust and highly sensitive. These biosensors are capable of detecting diverse ligands. Our biosensors have been applied to studies *in vivo* including clinical applications, highlighting the feasibility of this approach and the practicality of biosensors to detect specific molecules and biomarkers. What follows are descriptions of sensors developed under this proposal.

OVERVIEW

The ability to monitor biorecognition events and interactions on platforms offers pathways to the application of biological macromolecules as detectors. Coupled to the ability to precisely produce conductive elements on the nanoscale, biosensing offers unprecedented avenues for screening and detection at increasing sensitivities. Although biosensors have been an area of active investigation for a number of years, full realization of their potential has yet to be reached because the rates of reactions and sensitivities are significantly lower than in endogenous, biological systems. This deficiency can be due to the random nature of how the various signal transducing units are placed relative to the electrode. Consequently, integrating the precise three-dimensional information obtained from X-ray crystal structure analysis with nanotechnology platforms can result in highly enhanced systems. This gain is a consequence of

optimizing the geometrical parameters comprising the various components of the biosensor. Our lab developed an approach to rational production of biosensors by applying structural and conformational information of redox enzymes, peptides, and nucleic-acid based linker systems to production of biosensors, an integrated approach we call "coordinated biosensing". The fabrication of nanobiosensors, in our applications, consists of a biological molecule, linker or mediator, and nanoelectrodes. The various components can be equated with the electronic elements of a sensor as every module has to transduce the signals generated at the source (biomolecule) to the detector (electrode). Consequently, as in enzyme systems, rate improvements can occur from proximity and geometric effects, with potential enhancements of 10^2 to 10^3 at each junction. The additive consequence can be a gain of several orders of magnitude in rates with concomitant improvement in sensitivities.

We originally produced biosensors capable of detecting reactive oxygen species (ROS) and have refined the biosensors to detection of ROS generated from ambient ultrafine particles *in situ*. We have now expanded our repertoire to the detection of markers indicating metabolic status and certain disease states. In addition to development of metalized, peptide-based linkers, we have now produced and characterized other peptide / nucleic-acid based linkers. Concomitantly, we have expanded our protein detectors to numerous enzymes including NADH peroxidase, oxidase, glycerol kinase, glycerol-3-phosphate oxidase, and the membrane enzyme glycerol-3-phosphate dehydrogenase. We have integrated these biological sensing assemblies onto various electrode platforms, including carbon nanotube, gold nanoelectrode ensembles, gold disk, and wire electrodes. We have devised fully functional detection systems using the principles of coordinated biosensing, resulting in enhanced performance. These integrated sensors detected various markers at high sensitivities, demonstrating the feasibility of these as responsive detectors of biological signals. Our results indicate the potential applications of utilizing nanomaterials in detection, targeted cellular treatments and nanobiosensors in medical diagnostics to identify 'pro-disease' states. We have tested our biosensors for the detection of a cancer biomarker, prostate-specific antigen (PSA) and have now shown high specificity and sensitivity on patient serum samples. These current results confirm the value and applications of these biosensors.

COLLABORATIVE EFFORTS

We have collaborated with other PIs involved in this MURI project to more effectively integrate various expertise, reagents, and methodologies towards our collective goal of producing biosensors that can be integrated onto various platforms for ultrasensitive and specific molecular detection. Several of these are briefly listed below.

(1) Rod Beresford: integration of two biosensor assemblies, based on NADH peroxidase (ROS, H_2O_2 detection and quantification) and PSA-Ab (PSA detection and quantification), onto a flow-cell chip platform *(manuscript in preparation)*.

(2) Shana Kelley: integration of two biosensor assemblies, based on NADH peroxidase (ROS, H_2O_2 detection and quantification) and PSA-Ab (PSA detection and quantification) onto Au NEE nanoelectrode platform *(manuscript in preparation)*

(3) Jimmy Xu: collaborative design and integration of biosensor based on glucose oxidase; integration of two biosensor assemblies, based on NADH peroxidase (ROS, H_2O_2 detection and quantification) and PSA-Ab (PSA detection and quantification) onto CNT nanoelectrode platform *(two manuscripts published)*.

(4) Irwin Chaiken: design, characterization, and integration of Chaiken lab Zn-finger peptide linkers with various redox enzymes *(manuscript in preparation)*.

(5) Charles Martin: testing of nanomaterials produced by the Martin lab using NADH peroxidase and other oxidative enzyme electrodes for the production of reactive oxygen species. Cellular testing of the localization of these nanomaterials in RAW cells, using imaging approaches *(manuscript in preparation)*.

TECHNICAL DESCRIPTION OF BIOSENSOR / BIOASSEMBLIES

We have produced and fully characterized various linkers based on peptides and nucleic acids as well as protein detector assemblies. Our linkers and two of the bioassembly systems are described below; these have been fully integrated with various electrode and nanoelectrode platforms and onto micro flow-cells. These examples of bioassemblies delineate our approach and highlight the methodologies developed under this MURI proposal for the design and production of biosensors.

• Linkers – 'Bionanowires': Peptides and Nucleic Acid Analogs for Specific and Controlled Linkage of Proteins and Enzymes to Electrodes.

We have shown that leucine zippers can be designed to bind metals with controlled stoichiometries of metal binding and these serve as efficient electron transfer bridges that

additionally permit functionalization for specific linkage to targeted proteins. Functionality produces specificity of linkage, targeting enzymes at one end of the linker and the electrode surface at the other. To design functional groups to precisely bind to a specific region of the enzyme, we use the three-dimensional structure. These coordinates provides atomic resolution details of exactly where each residue of the protein is and whether mutating or linking a particular residue to the linker would affect function or fold. Using this structural-based approach, we designed metalized-histidine peptides ('MHP'), which are capable of binding divalent metals and of sufficient helical length and constrained geometrical configuration to penetrate the active sites of target enzymes. These

MHPs can conduct electronic signals generated at the active site to electrode surfaces. We have expanded our peptide library and have now produced peptide-nucleic acid (PNA)-based linkers. We have characterized these through MS, SPR, and atomic-resolution structure determination of several PNAs of differing sequences and metal-ligation sites. Consequently, our linker collection is composed of numerous peptides and PNA that can be functionalized to specifically link to various protein and redox enzymes, to bridge the active sites of proteins and electrode surfaces. In addition to these peptide and nucleic acid based linkers, we have, in

collaboration with the Chaiken group, characterized several of their coiled-coil peptides as additional linker systems.

• Biosensors for the Detection and Quantification of Oxidative and Metabolic Compounds

We reported, starting in 2004, the production of a fully-active and functional enzyme bioassembly immobilized onto gold electrodes using a new peptide linker system. We developed this system for detection of reactive oxygen species (ROS) through the function of the redox enzyme, NADH peroxidase (Npx). In 2005 and 2006, we continued to characterize this system by isothermal titration calorimtery (ITC), surface plasmon resonance (SPR), mass spectrometry (MS), X-ray absorbance fluorescence spectroscopy (EXAF), and X-ray crystallography, examining each component of the system to develop a theoretical foundation for the enhanced performance of the biosensor. Through electrochemical approaches including cyclic voltammetry (CV) and chronoamperometry, we were able to correlate how various parameters affected performance of each component, resulting in a rational approach to biosensor design that we called "coordinated biosensing". As reported in 2006, we successfully integrated these bioassemblies onto carbon nanotube electrode (CNT) arrays (in collaboration with the Xu group), resulting in enhanced overall performance. We also integrated these bioassemblies onto Au nanoelectrode ensembles (NEEs; collaboration with the Kelley group). In 2007, we started to characterize NEE platforms with the Npx system as well as other fully functional enzyme/protein-based systems (described in previous progress summaries). As reported in previous reviews, we expanded our enzyme and biolinker 'libraries' and now have fully-functional biosensors capable of detecting ATP, glycerol, and targeted metabolomic compounds that indicate the specific metabolic, energy, and cellular status of cells. We have been able to fabricate biosensors capable of detecting cancer biomarkers and have recently tested these on patient serum samples, proving that these biosensors can be used in biological milieu and *in-situ*.

• Biosensors for the Detection of Clinically-Relevant Biomarkers: PSA biosensor

We have expanded our protein/enzyme library to detect numerous markers, including those important for toxicological applications and in detecting abnormal cellular states. Our structural work in the area of glycerol metabolism enzymes provides the detailed conformational information for optimization of the macromolecular components in these sensors. In conjunction with designed linkage to nanoelectrodes, using our bionanowires described above, arrays of amperometric nanobiosensors have been fabricated to detect a diverse array of compounds. The sensitivity and specificity of the sensors are based on endogenous recognition motifs and native mechanisms of enzymes, in principle. We have furthered this objective by producing biosensors capable of monitoring metabolic cellular status and abnormal (disease) states. For the prostate-specific antigen (PSA), we have achieved detection levels that are comparable or surpass currently used assays, by application of our coordinated biosensing approach (table below). This system detects abnormalities on a two-tiered detection system, augmenting the capabilities of the biosensor. The table below compares the various detection approaches.

Biosensor type	Detection Limit	Reference
PNA/PSAantibody/PSA	0.01 ng/mL	Yeh Lab
Quantum dots (CdSe@ZnS)	0.02 ng/mL	Lin Y, et al., Biosen. Bioelectron. 2008
Multi-walled carbon nanotubes	0.08 ng/mL	Panini N, et al, Biosen. Bioelectron. 2007
Single-walled carbon nanotubes	0.25 ng/mL	Okuno J, et al., Biosen. Bioelectron. 2007
Sandwich immunoassay	0.25 ng/ml	Sarkar P, et al., International Journal Pharmaceutics. 2002
Sol-gel film	3.4 ng/mL	Liu Y, Thin Solid Films. 2007

• **Single Molecule Biochemistry and Detection of Enhanced Enzymatic States**

We have continued to look at fundamental biochemistry of single macromolecules, to identify conformations of enhanced enzyme reaction states. This follows on advances in single-molecule enzymatic assays for characterizing single molecule kinetics. Previous models based on ensemble averaging are insufficient to explain rates of reactions as the ability to measure single molecule properties become feasible. The distributions and fluctuations in molecular properties can now be characterized and as techniques advance, key transient states can be identified. As the identification of highly efficient conformational states of enzymes can have profound implications in the design of sensors, we have been investigating the detection and modeling of conformational states of redox enzymes through predictive computational algorithmic training approaches previously utilized in our laboratory. This area additionally integrates our high-resolution structural studies to provide insight into identifying, on a predictive basis, the macromolecular conformational states that are more advantageous for enhanced signal transduction. As applied to the objectives of this project, utilizing enhanced states of enzymes can provide innate improvement in sensitivities and kinetics.

OBJECTIVES REACHED

- Production and characterization of a library of peptide and PNA linker as bionanowires.
- Integration of bionanowires with enzymes and electrode surfaces for formation of fully functional biosensors, capable of detecting toxicological and cellular markers.
- Construction of nanobiosensors using CNT as nanoelectrode platform (collaboration with Xu group).
- Characterization of nanobiosensors using Au NEEs as nanoelectrode platform (collaboration with Kelley group).
- Integration of bioassemblies onto micro flow-cell platform (collaboration with Beresford group).
- Studied enhanced single molecule states through combination of computational modeling and experimental approached (FRET, EXAF, X-ray crystallography) to identify enhanced functional states.

CONCLUDING RESULTS (SUMMARY OF ABOVE OBJECTIVES)

- Completed integration of linker library and enzyme library with nanoelectrode platforms to produce biosensors with broad substrate detection capability.
- Characterized biosensors *in-situ* on cells and in patient serum showing sensitivity and specificity of biosensors. Application of nanobiosensors for *in-situ* and *in-vivo* detection on cellular systems and clinical samples are clearly feasible.

Interfacing Biomolecules and Electroactive Nanomaterials

Shana O. Kelley

Development of nanostructured microelectrode arrays for multiplexed analysis of biosensor assemblies. One focus of our efforts in the past year has been the development of a new multiplexed electrode platform. We desired to fabricate a device that would have the following features: *1)* it would contain individually addressable electrodes with nanoscale features that would promote diffusional reactions leading to high biological-to-electronic signal transduction efficiencies, *2)* it would feature an ideal electrode material that would facilitate attachment chemistry and fast electron transfer from sensor assemblies to the chip, and *3)* it would be multiplexed and scalable. After iterating through several chip designs that failed to meet one of these criteria, we have now identified an ideal type of device for multiplexed studies. The chip features nanostructured microelectrodes (NMEs); an example of a NME is shown in Figure 1. A chip containing multiplexed NMEs has been prepared and used to test the NADH peroxidase biosensors prepared by the Yeh group. As shown in Figure 2, this chip yields large signals, and interestingly, much larger turnover numbers are obtained relative to those measured on a bulk gold electrode. This finding underscores the importance of nanostructuring in facilitating bioelectro-chemical reactions. The presence of the small features displayed on NMEs allows substrates to access the enzyme assembly by radial diffusion, which promotes high levels of reactivity. Two papers about the NME chip are currently submitted, both crediting the MURI grant for support.
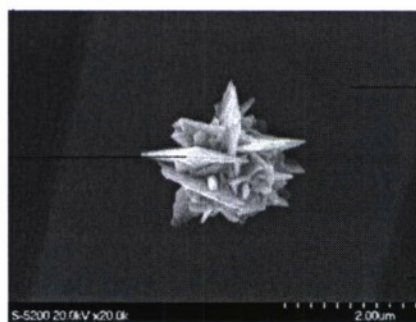
Figure 1. Nanotextured microelectrode

Turnover comparison:

Mocro Au: 200 s^{-1}

NEE: 3600 s^{-1}

NME: 5000 s^{-1}
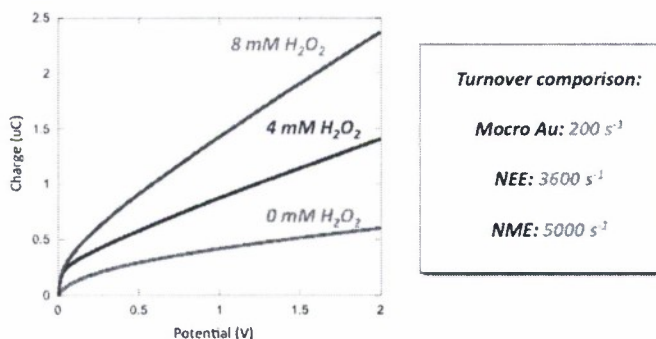
Figure 2. Response of NADH peroxidase enzyme assemblies at collected on a NME chip.

Development of electrochemical system for assessing conductivities of coiled-coil peptide linkers. In collaboration with the Chaiken group, we have investigated a series of coiled-coil assemblies as electrically conductive linkers for biosensor assemblies.

Significant advances have been made in the development of electrochemical assays that confirm the immobilization of complexation of the Chaiken group peptides. As shown in Figure 3, we are able to monitor immobilization of one coil, and then observe the complexation of the second using a simple electrochemical assay. We have also attempted to monitor electrochemical signals flowing to an appended redox reporter group to judge the conductivies of the assemblies. However, this has proven to be challenging, and we were not able to observe electron flow through these assemblies using electrochemistry.
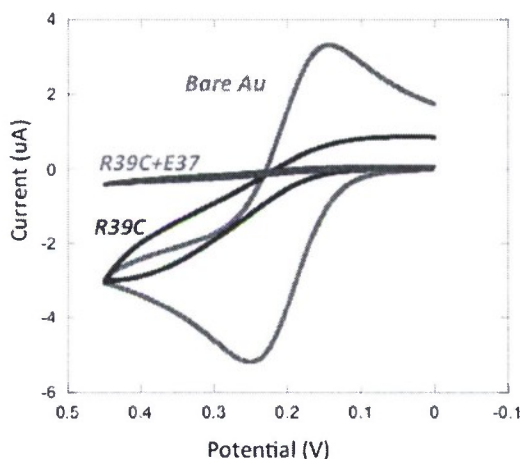
Figure 3. Visualization of coiled-coil assembly using ferrocyanide blocking.

Exploiting CNT-DNA interactions for nanomaterials separation. In collaboration with the Gilman group at NIST, we are working towards an optimized system for CNT separations. Over the last year, we participated in a study spearheaded by Gilman where a variety of agents were compared for CNT solubilization. Our contribution to this effort was to design and synthesize a variety of DNA sequences, and interestingly, it was these agents that were some of the most effective that were studied. A paper documenting these funding was published in 2008 in Langmuir.

Designed Biomolecular Interfaces and Linkers as Enabling Technology for Developing Advanced Biosensors

Irwin Chaiken

At the outset of this program, several overarching efforts were identified for the Drexel Group. These are listed as follows, with the first being the main goal and the other two more minor.

- Task 1: Design of dimeric coiled coil and self-assembling modular miniprotein scaffolds that will contain components for linking the sensor surface of carbon nanotubes to biological targets.
- Task 2: Incorporation of conducting metals into miniproteins to produce conducting linkers.
- Task 3: Incorporation of sequences and other chemical groupings onto miniprotein modules in order to adapt these to DNA, and to other linker compounds and to reactive groups on nanotubes.

OVERVIEW OF ACHIEVEMENTS

Achievements for Task 1: Self-Assembling Linker Development

Track 1. We designed a series of coiled coil peptide systems as linker candidates and established their kinetics of self-assembly. Heterodimer anchor-probe coiled coils were designed and constructed. Linkage of anchor to sensor surfaces was achieved. Kinetics of assembly of probe module to anchor was determined using surface plasmon resonance sensing.

Track 2. We demonstrated that modular coiled coil heterdimers could be assembled specifically on CNT tips. Methodologies were developed and implemented for linkage using disulfide and triazole covalent attachment. Scanning electron microscopy (SEM) imaging of coiled coil assemblies was achieved.

Achievements for Task 2: Metallization and Conductivity

We demonstrated that coiled peptide modules with metal binding sites and fused redox centers can be used to form heterodimer coiled coil linker interfaces on sensor surfaces. Cobalt adducts were designed using His residues. Gold adducts using Cys residues were derived. Ferrocene conjugates of coiled coil modules were constructed. Assembly on gold electrodes was validated (with Kelley lab). Covalent fusion of coiled coil modules with NADH peroxidase (NPX) was tested (with Yeh lab). Conductivity testing was carried out (with Kelley and Yeh labs).

Achievements for Task 3: Expansion of Probe Recognition Capabilities

A recombinant expression system for the production of E-coils in bacteria was created. Using auxotrophic expression, methionine residues encoded in the sequence were replaced with the non-canonical amino acid, homo-azidoalanine. Site directed mutagenesis was employed to alter the encoded E-coil sequence, placing a chromophore for accurate determination of concentration by spectroscopic means.

DETAILS OF ACHIEVEMENTS

Achievements for Task 1: Self-Assembling Linker Development
Establishing self-assembling and regenerable linker systems for interfacing biomolecules with carbon nanotube (CNT) surfaces was the major goal of the Drexel project in this MURI program. Two major track of work were pursued, (1) design of self-assembling linkers and (2) establishment of methods for controlled attachment to CNTs.

Track 1. We designed a series of coiled coil peptide systems as linker candidates and established their kinetics of self-assembly. Heterodimer anchor-probe coiled coils were designed and constructed. Linkage of anchor to sensor surfaces was achieved. Kinetics of assembly of probe module to anchor was determined using surface plasmon resonance (SPR) sensing.

At the outset of this work, we had in hand prototype homo- and hetero-dimer coiled coil pairs and determined their stabilities using circular dichroism measurements. Heterodimers were seen as the key tools for developing self-assembling modular linkers on sensors (**Fig.1**). Hence we adapted the heterodimer system for sensor immobilization. One of the components, the positively charged heptad repeat peptide "R-coil", was modified to enable sulfhydryl-directed attachment to CNTs. This linkage chemistry was established using surface plasmon resonance technology and then adapted for the CNT application (see below). R-coils were denoted "anchors".
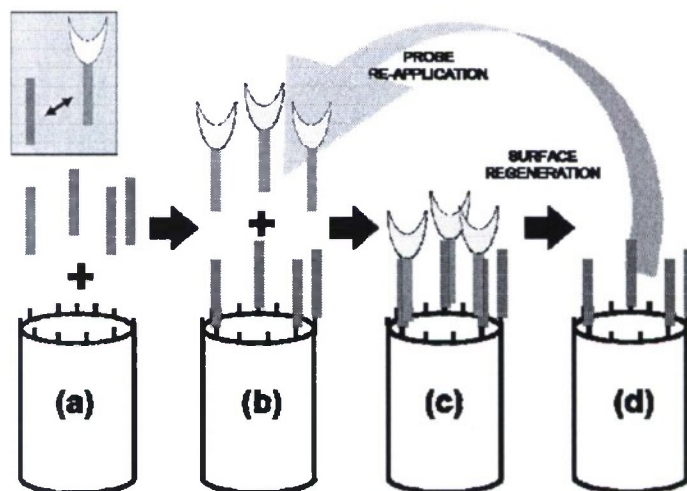
Figure 1. Self-assembly and regeneration of a CNT sensor surface using an anchor/probe strategy. (Inset) The blue anchor is able to non-covalently recognize the red probe that can be fused to a cyan biological component. (a) Oriented covalent attachment of the anchor to the CNT tips enables a recognition surface for the probe. (b) Upon addition of the probe in solution, self-assembly is able to isotropically display (c) the cyan target molecule through the capture component (red). The biological component is free to access the solvent and its native target. (d) Upon addition of a regeneration solution that disrupts biological assembly between the anchor/probe, the probe is washed from the surface and can be reapplied when desired for further experimentation.

The other module of the self-assembling coiled coil heterodimer was the negatively charged or E-coil. This was designed to interact with the R-coil *via* charge complementarity and hence to

assemble tightly but reversibly to R-coils anchored to sensor surfaces. E-coils were designed to contain covalently fused polypeptide, protein or other molecular components that either by themselves or by non-covalent binding would contain signal-generating structures. The E-coils were defined as "probes". The interaction kinetics of immobilized anchor modules and soluble probe modules was measured by surface plasmon resonance. An example of R-coil anchor, E-coil probe and kinetics of interaction between them is shown in **Figs. 2-4.**

R39H10

Figure 2. Characteristics (left), RP-HPLC (top right) and mass spectrogram (bottom right) of the anchor coil R39H10. The major elution peak at 26.5 minutes was found to correspond to a mass of 4773.2, which is close to the expected mass of 4771.6 Daltons.

E37Ferro

Figure 3. Characteristics (left), RP-HPLC (top right) and mass spectrogram (bottom right) of the probe coil E42C. The major elution peak at 21.5 minutes was found to correspond to a mass of 4550.6, which is close to the expected mass of 4550.8 Daltons.

Figure 4. (left) Characteristic sensorgram of E37Ferro over immobilized R39H10 and (right) a plot of the residuals used to evaluate the fit of the 1:1 Langmuir global interaction model.

The kinetics of interaction of a set of anchor and probe components as determined by SPR is given in **Table 1**.

Anchor/Probe		R39C		R39H10		R39Pra*	
E42C	k_{on} [M^{-1} s^{-1}]	3.27E+05	± 0.97	4.01E+05	± 0.86	1.00E+05	± 1.59
	k_{off} [s^{-1}]	1.64E-04	± 0.99	2.23E-04	± 0.64	3.23E-04	± 1.60
	K_D [M^{-1}]	5.0E-10		5.6E-10		3.23E-09	
E37Ferro	k_{on} [M^{-1} s^{-1}]	4.24E+05	± 0.44	4.77E+05	± 0.57	1.57E+05	± 1.07
	k_{off} [s^{-1}]	1.42E-04	± 0.86	2.35E-04	± 0.17	6.18E-04	± 0.98
	K_D [M^{-1}]	3.3E-10		4.9E-10		3.9E-09	

Table 1. Kinetic parameters acquired by surface plasmon resonance biosensing. Standard deviations were obtained from a minimum of three experiments, while K_D was calculated as the ratio of k_{off} to k_{on}. *The anchor R39Pra did not fit the global 1:1 Langmuir binding model used with R39C and R39H10; local fits were used to estimate these parameters.

We established that the self-assembly of modular anchor-probe heterodimer coiled coils was anti-parallel. This was carried out by fluorescence analysis as shown in **Fig. 5**. This type of orientation guided the design of modular self-assembling linkers in the project.

Figure 5. Different alignments of R39C (blue) and E42C (red) would place free thiol groups away (parallel) or next to one another (anti-parallel). These alignments would dictate the self-assembly of larger proteins (cyan) on sensor surfaces that were fused to the probe coil E42C. Our hypothesis was to test parallel versus anti-parallel formation in our dimer coiled coil using a FRET assay. The data did not demonstrate any fluorescence quenching, which supported a parallel formation.
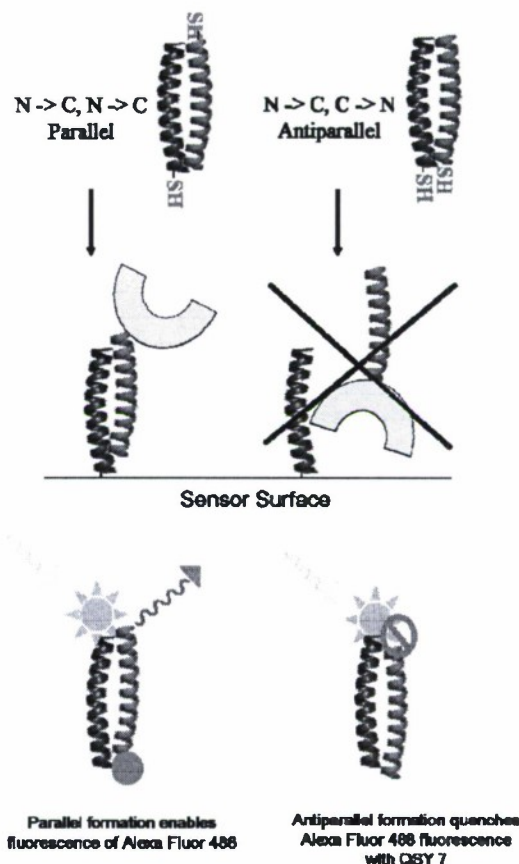
We established that heterodimer sensor interfaces can be formed with E-coil probes containing fused biomolecular recognition components, and that such fused components remained accessible and functional. This was demonstrated with an E-coil containing a His tag. This probe was associated with an R-coil linked to an SPR sensor surface, and bound probe was subsequently associated with anti-His tag antibody as analyte (**Fig. 6**).

Figure 6. Kinetic analysis of anti-pentaHis monoclonal antibody captured via an assembled anchor/probe combination displaying a terminal 6XHis motif. Concentrations of the antibody are as follows: blue 5 nM, red 10 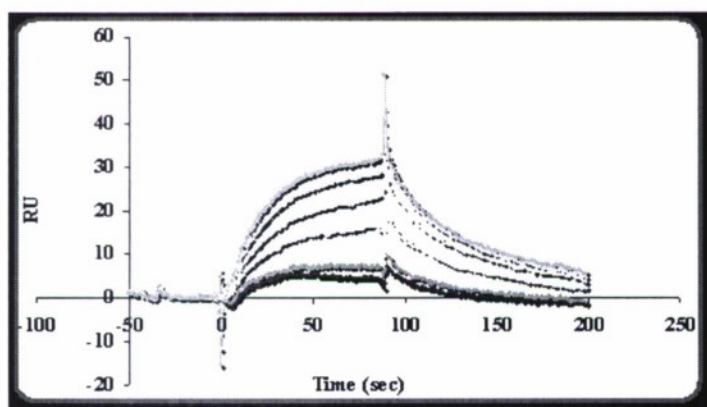nM, yellow 25 nM, cyan 50 nM, green 75 nM, and orange 100 nM. Fitting the sensorgrams to a global 1:1 Langmuir model resulted in a nanomolar affinity (Kd = 49.0 nM +/- 4.2).

Track 2: We demonstrated that modular coiled coil heterdimers could be assembled specifically on CNT tips. Methodologies were developed and implemented for linkage using disulfide and triazole covalent attachment. Scanning electron microscopy (SEM) imaging of coiled coil assemblies was achieved.

Two types of chemistries were developed for attachment of R-coil anchors to CNT tips. These are depicted in **Figure 7** below. The second of these was developed to insure that, in multiplexed amperometric applications of the coiled coil linker, the anchor would remain attached to the CNT sensor in sequential rounds of probe-anchor assembly, redox reactions and regenerations.
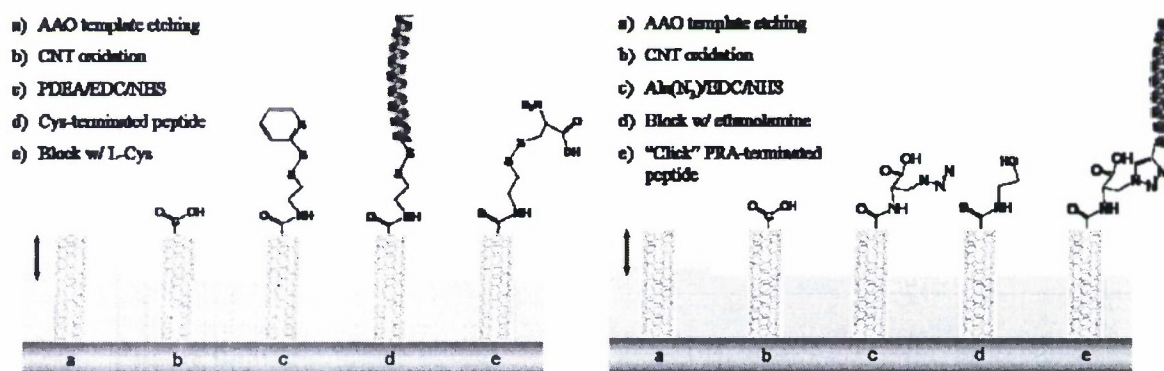
Figure 7. Left) schematic diagram illustrating the sequence of functionalizing a CNT array with the R39C coiled coil anchor using a disulfide exchange reaction. (a) Template etching and oxidation (b) are used to create reactive carboxyls. (b) Solution applied EDC/NHS/PDEA chemistry allows for covalent attachment of anchor peptides through a mixed disulfide reaction. (e) Residual pyridyldithiol sites are blocked with a cysteine-salt solution. Right) Schematic diagram illustrating the sequence of functionalizing a CNT array with the R39Pra anchor using a 1,3 Huisgen dipolar cycloaddition to form a 1,2,3 triazole. (a) Template etching and oxidation (b) are used to create reactive carboxyls. (c) Applied EDC/NHS/homo-azido alanine presents a reactive azide; (d) residual sites are blocked with an ethanolamine solution. (e) Covalent immobilization was achieved with a copper-catalyzed 1,2,3 triazole formation between the azide of homo-azido alanine and the alkyne of R39Pra.
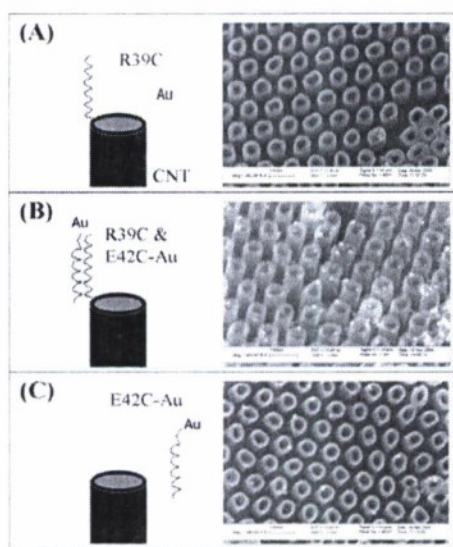
Figure 8. Disulfide exchange immobilization of the anchor R39C coiled coil and E42C assembly onto highly ordered CNT arrays. (A) SEM image of the covalent immobilization of anchor peptide R39C in H_2O onto etched tips of the CNT array and unconjugated nanoparticles. (B) Upon addition of the Au-labeled E42C probe peptide again in H_2O, the tips of the carbon nanotubes demonstrate site-specific assembly of the coiled coil peptides. (C) Without the covalent attachment of the anchor peptide, Au-labeled E42C show minimal non-specific adsorption to the CNT array following simple substrate washing with H_2O. The control image of unlabeled Au nanoparticles and R39C indicates interaction is specific between R39C and E42C, not R39C and nanoparticle.

Assembly of soluble E-coil probes to R-coil anchors attached to CNTs was established with gold-labelled E-coils, using SEM imaging to track interactions and their specificity. An example of this process using disulfide linkage is shown in **Fig. 8**.

Figs. 9-11 depict the demonstration for forming self-assembling linkers using triazoles.
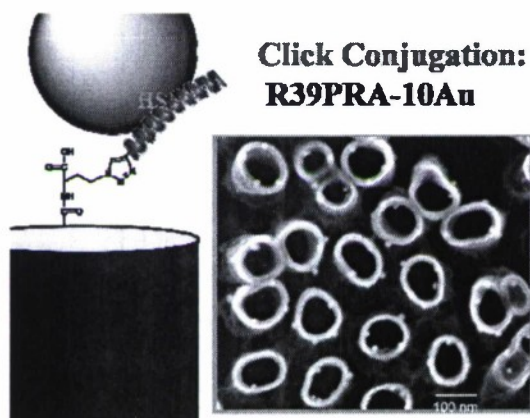
Click Conjugation: R39PRA-10Au

Figure 9. Click conjugation of the anchor peptide R39Pra to the tips of a CNT array. The thiol located on the central heptad was labeled with 10 nm gold nanoparticles to provide SEM contrast.

Click to CNTs: Controls

Figure 10. Controls for the click chemistry attachment of the anchor peptide R39Pra. In the top image the CNT array did not have the homo-azido alanine functionality on the tips, all other steps of the click immobilization pathway were retained. In the bottom image, the R39Pra anchor was immobilized to CNT tips, but the central thiol was blocked with 1 M iodoacetic acid before incubation with 10 nm gold nanoparticles.
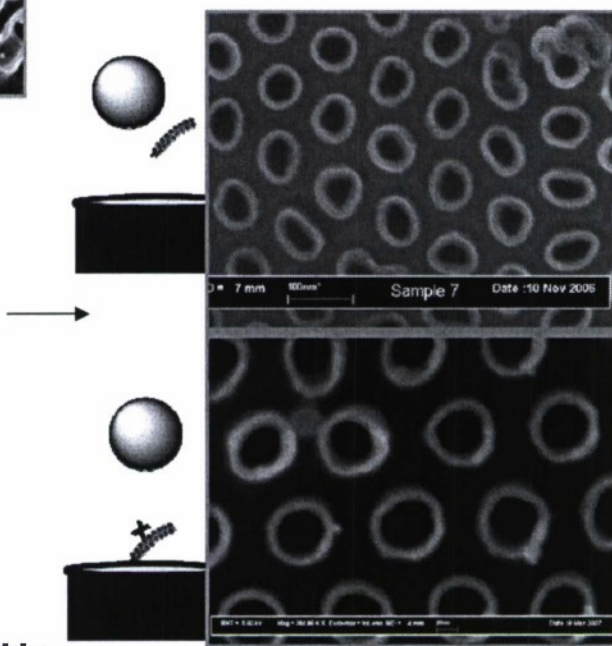
Click Assembly: R39PRA-blocked Cys18 & E42C-20Au

Figure 11. Assembly of E42C labeled with 20 nm gold nanoparticles onto a clicked anchor peptide R39Pra with a blocked thiol group.

Achievements for Task 2: Metallization and Conductivity

We demonstrated that coiled peptide modules with metal binding sites and fused redox centers can be used to form heterodimer coiled coil linker interfaces on sensor surfaces. Cobalt adducts were designed using His residues. Gold adducts using Cys residues were derived. Ferrocene conjugates of coiled coil modules were constructed. Assembly on gold electrodes was validated (with Kelley lab). Covalent fusion of coiled coil modules with NADH peroxidase (NPX) was tested (with Yeh lab). Conductivity testing was carried out (with Kelley and Yeh labs).

We identified the tolerance of coiled peptides for multiHis substitutions and metallization by Co++ with retention of heterodimer formation between anchor and probe modules. The ability of multi-His peptides to bind Co++ was confirmed by immobilized metal affinity chromatography (IMAC), isothermal titration calorimetry (**Fig. 12**) and mass spectrometry. ITC and SPR confirmed that cobalt-bound peptides were able to form stable coiled coil heterodimers. Interaction kinetics for R39C anchor and E42C by SPR are shown in **Table 2**.
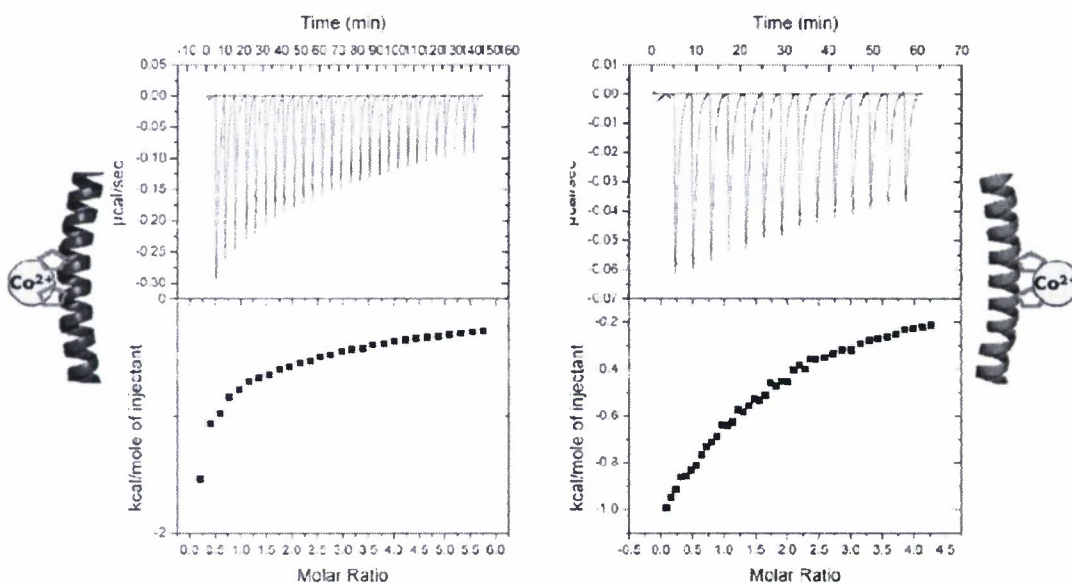
Figure 12. Isotherms of cobalt titrated into R39C (left) and E42C (right). Peptide concentrations were determined via UV absorbance, whereas cobalt concentration was determined by weight and subsequent dilution in HBS-P. The affinity of the cobalt ion for each peptide was estimated to be ~10 - 100 µM with a stoichiometry of ~ 0.8 - 1. The single set of sites model was unable to accurately predict these values using the Origin software.

BUFFER		1:1 with Mass Transfer with Cysteine Reference (fc1)					
		1	2	3	4	avg	stdev
Normal	k_{on} (1/Ms)	3.15E+05	2.77E+05	3.02E+05	3.63E+05	**3.14E+05**	± 0.36
	k_{off} (1/s)	3.68E-05	2.11E-06	1.65E-07	1.88E-07	**9.82E-06**	± 0.18
	K_D (M)					**3.12E-11**	
Metallized	k_{on} (1/Ms)	3.62E+05	2.34E+05	1.53E+05	9.25E+05	**4.19E+05**	± 3.48
	k_{off} (1/s)	9.52E-05	8.27E-05	5.29E-05	2.74E-04	**1.26E-04**	± 1.00
	K_D (M)					**3.02E-10**	
		1:1 with Mass Transfer with 100 RU Interleukin 5 Reference (fc2)					
		1	2	3	4	avg	stdev
Normal	k_{on} (1/Ms)	2.17E+05	3.80E+05	2.80E+05	4.32E+05	**3.27E+05**	± 0.97
	k_{off} (1/s)	4.04E-04	2.30E-07	2.52E-04	1.19E-08	**1.64E-04**	± 0.99
	K_D (M)					**5.01E-10**	
Metallized	k_{on} (1/Ms)	1.59E+05	2.01E+05	1.65E+05	1.80E+05	**1.76E+05**	± 0.19
	k_{off} (1/s)	6.29E-05	1.63E-04	1.24E-04	3.16E-04	**1.66E-04**	± 1.08
	K_D (M)					**9.45E-10**	
		90 minute Dissociation with Cysteine Reference (fc1)					
		1	2	3	4	avg	stdev
Normal	k_{off} (1/s)	1.79E-04	1.71E-04	1.04E-04	n.d.	**1.51E-04**	± 0.41
Metallized	k_{off} (1/s)	2.28E-04	3.16E-04	2.15E-04	n.d.	**2.53E-04**	± 0.55

Table 2. Summary of SPR acquired kinetic parameters in normal and metallized conditions. Association (k_{on}) and dissociation (k_{off}) constants were obtained via double referencing from both cysteine and non-specific protein, interleukin 5 (IL5), surfaces globally fitting to a 1:1 Langmuir interaction model corrected for mass transfer. 0, 10, 20, 50 and 100 nM E42C solutions in normal and metallized buffers (HBS-P / HBS-P with 1mM $CoCl_2$ respectively) were allowed to accumulate for 180 seconds and dissociate for 360 seconds. Equilibrium constants (K_D) were calculated from k_{off} / k_{on} using the average values determined in the table. For the 90 minute dissociation fits, the dissociation constant (independent of concentration) was obtained with a 50 nM injection of E42C using the cysteine reference surface. n.d. stands for not determined.

Assembly of metallized peptides was demonstrated on macroscale and nanoscale gold electrodes using cyclic voltammetry (Yu and Kelley lab). Evidence for formation of dimers was obtained by demonstrating the presence of ferrocenyl-probe E-coil peptide on electrodes to which R-coil was attached through a Cys SH group. A demonstration of this is given in **Fig. 13.**

Several attempts were made to observe conductance of electrons through metallized heterodimers from redox centers fused to E-coil components to an amperometric sensor containing R-coil fused to the electrode. This work led to largely negative results. Redox centers examined included ferrocene (with Kelley lab) and Npx (with Yeh lab). An example of the results obtained is shown in **Fig. 14**.

In collaboration with the Yeh Lab, a positive though weak conductance signal was obtained with a single, highly ruthenioum-metallized R-coil containing a fused anti-PSA antibody and attached covalently to a macro gold electrode. As shown in **Fig.15**, conductance was observed upon addition and apparent binding of a PSA-glucose oxidase chimera.
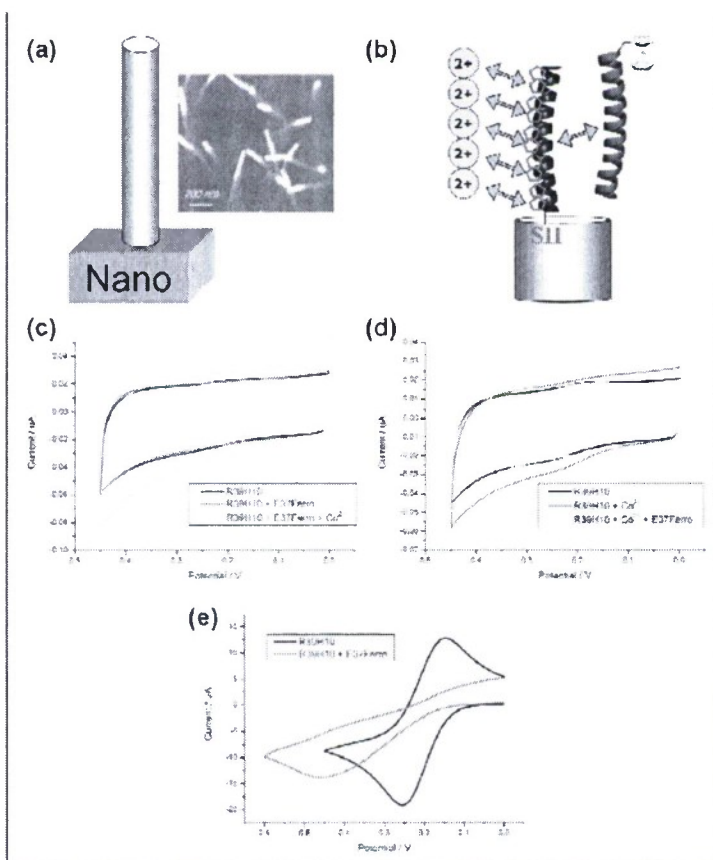
Figure 13. Cyclic voltammetry of R39H10 and E37Ferro on nanoscale gold electrodes. (a) Schematic depiction of a gold nanopost and SEM image of the NEE electrode before function-alization. (b) Schematic diagram of the peptide assembly in which R39H10 is anchored via a C-terminal thiol to the gold electrode, while E37Ferro is captured in the presence and absence of a divalent cation. (c) CV scans of R39H10 alone, R39H10 and E37Ferro com-plexes, and the subsequent addition of 1 mM $CoCl_2$ to these complexes. (d) CV scans of R39H10, R39H10 with 1 mM $CoCl_2$, and R39H10-Co^{2+} with E37Ferro. (e) CV scans of R39H10 and R39H10-E37Ferro in the presence of 2 mM ferrocyanide. All CV scans were carried out in 25/25 buffer with a scan rate of 100 mV/s.
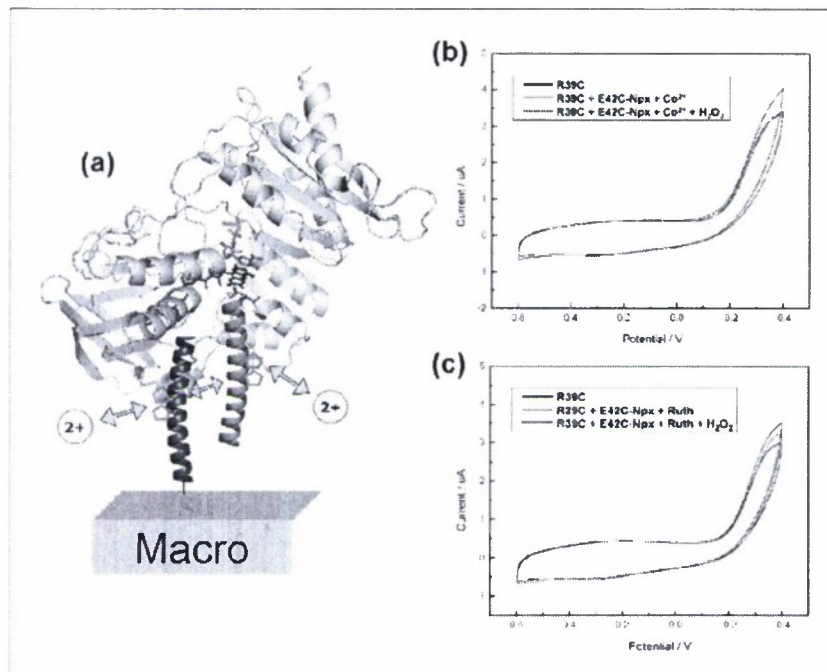
Figure 14. Cyclic voltammetry of R39C and E42C-Npx construct on macroscale gold electrodes. (a) Schematic diagram of the peptide assembly in which R39C is anchored via a C-terminal thiol to the gold electrode and is able to capture E42C-Npx in the presence and absence of a divalent cation. (b) CV scans of assembled peptides in 1 mM $CoCl_2$. (d) CV scans of assembled peptides in the presence of 1 mM $(Ru(NH_3)_6)Cl_2$ and 100 μM H_2O_2. All CV scans were carried out in KAc (pH 5.4) buffer with a scan rate of 100 mV/s.
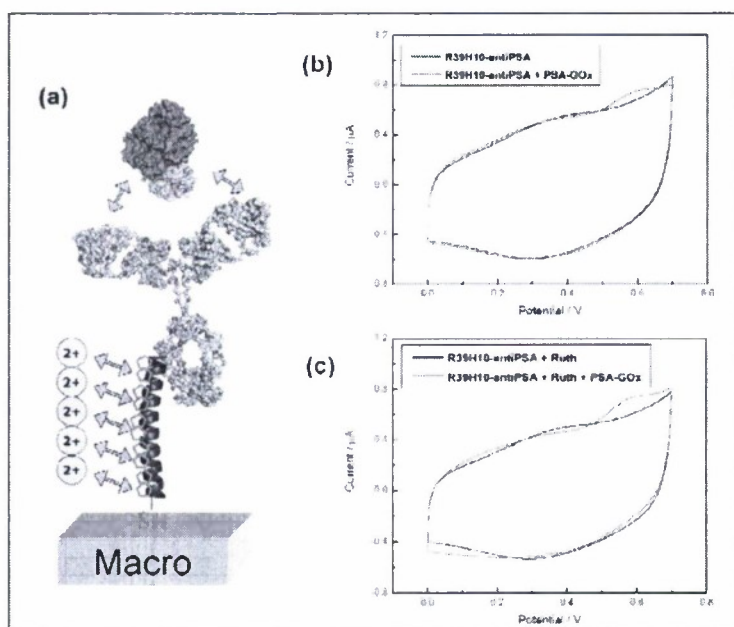
Figure 15. Cyclic voltammetry of R39H10-antiPSA and PSA-GOx construct on macroscale gold electrodes. (a) Schematic diagram of the peptide assembly in which R39H10 is anchored via a C-terminal thiol to the gold electrode covalently linked to the antiPSA antibody, enabling capture of the PSA-GOx (green-magenta) construct. (b) CV scans of assembled biosensor in the presence and absence of PSA-GOx target. (c) CV scans of biosensor in the presence of 1 mM $(Ru(NH_3)_6)Cl_2$ and 100 μM H_2O_2. All CV scans were carried out in KAc buffer (pH = 5.4) with a scan rate of 100 mV/s.

Achievements for Task 3: Expansion of Probe Recognition Capabilities

Several achievements were made to incorporate sequences and other chemical groupings onto miniprotein modules that could ultimately be used to expand adaptability of the linkers to interface reactive groups on nanotubes and biological targets. Work on this task was integrated into the work of Tasks 1 and 2. Achievement highlights 1-3 are as follows.

1. A recombinant expression system for the production of E-coils in bacteria was created. Purification was carried out with metal chelate chromatography and size was verified by gel electrophoresis (**Fig. 16**). This recombinant probe can be fused to proteins of interest with a sequence that was shown to assemble with high affinity to the anchor sequence. Attempts to fuse this sequence to NPx and GOx were unsuccessful at the protein expression stage.
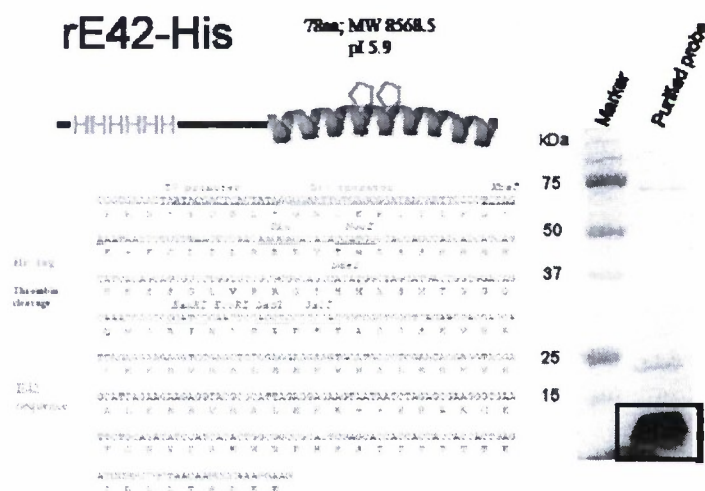
Figure 16. Characteristics (left), sequence (bottom left) and SDS Page gel (right) of the probe coil rE42-His. The vector encoding rE42-His was expressed in E. coli and purified using immobilized metal affinity chromatography and resulted in a large band at the desired molecular weight of roughly 9 kDa.

2. Using auxotrophic expression, methionine residues encoded in the sequence were replaced with the non-canonical amino acid, homo-azidoalanine (Fig 17). This pathway affords a means to insert functional groups for selective reaction to nanoparticles and electron mediators such as ferrocene using a "click" chemistry pathway.

rE42-A(N₃)-His
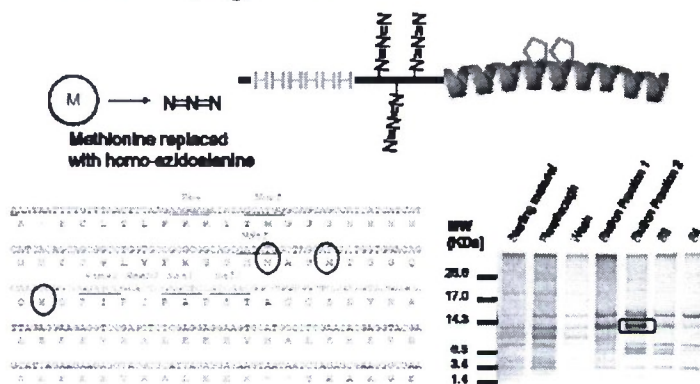
Figure 17. Characteristics (top), sequence (bottom left) and SDS Page gel (bottom right) of the probe coil rE42-A(N₃)-His. The vector encoding rE42-His was expressed in methionine auxotroph E. coli using and purified using immobilized metal affinity chromatography and resulted in a band at the molecular weight of roughly 11 kDa. Elution Fraction 2 resulted in the highest amount bound by SPR analysis.

3. Site directed mutagenesis was employed to alter the encoded E-coil sequence, placing a chromophore for accurate determination of concentration by spectroscopic means. This technique can be further paired with auxotrophic expression to encode methionine residues at specific loci within the E-coil probe.

Photosynthetic Reaction Center:
Optical Switch, Photoactuator, and Photosensor

Nikolai Lebedev

This section summarizes the effort and accomplishments of the Lebedev group. It is organized in accordance with the initial statement of work of this part of the MURI project exactly as it was planned at the outset of the program.

Specific task 1. Develop principles and methods for vectorial binding of photosynthetic reaction center (RC) to carbon electrode (year 1) (see "Summary of the constructed mutants" at the end of this section.)

1. RC cloning, expression, and purification. Constructed the bacterial host and expression cassettes allowing for performing site-directed modifications in any RC subunits (L, M, and H). Demonstrated the feasibility of mutant construction in all of these subunits. Demonstrated high the efficiency of the strategy of the purification of any of the constructed proteins on amylose column. The developed strategy allows for the production of any constructed protein mutants up to several mg of pure protein within few days.

2. Construction of site-specific linkers for oriented binding of photosynthetic RC (R.sphaeroides) to carbon electrodes (grids and CNT) using Cys and 7His. Constructed RC double and triple mutants that in addition to the specific modification inside the protein have either poly His at the P-side, or Cys at H-side or both poly His and Cys mutations.

3. Demonstration of optical switching effect for ET between RC and carbon electrode. All the constructed protein mutants are tested for the possibility of photoinduced electron transfer between the protein and carbon electrode (as well as gold electrode) in protein orientation-dependent manner.

4. Construction of R.capsulatus mutants with different Cys locations. Test the effect of Cys location on the efficiency of RC binding and the possibility of direct electron transfer to the electrode. Constructed RC Cys mutants with one, two and three Cys at the LM side facing H-subunit. In addition, in some of them internal Cys L92 was substituted with Ala.

5. Test various mobile carriers and mobile phases for electrical connection of RC to CNT. Several quinones with alkane tails of various lengths soluble either in water or in hydrophobic solvents were tested for the best electron transfer activity in mobile phase. The optimal conductance for aquase solutions were found with Q2.

In general, the result of these work led to the development of the strategy for the construction of RC protein monolayer on various electrodes, including carbon, gold, ITO, and CNT with total surface coverage about 75-80%. The constructed surfaces were highly efficient in photoinduced charge separation and electron transfer to or from modified electrode (depending on protein orientation). The parameters and controlling factors of electron transfer between RC protein and the electrode were estimated and low reorganization energy and adiabatic distance about 10-12 Å were identified (**Biosensors & Bioelectronics** 19:1649-1655, 2004, **Biosensors &**

Bioelectronics 21:1023-1028, 2006, **Journal of Physical Chemistry-C**, 111:17122-17130, 2007; Alan Berman Research Publication award of 2008). These efforts formed a background for our construction of a solid-state PV device based on photosynthetic RC proteins (**NANO Letters** 4:1079-1083, 2004).

Specific task 2. Construction of a stable heteroprotein ET complex at the donor side of RC (year 2)

1. Identification of amino acids controlling RC-Cyt binding (by computer modeling and SDM)
2. Construction of bifunctional linkers for specific domains in RC and the other ET proteins (cytochrome or glucose oxidase)
3. Construction and incorporation of conductive linker in the constructed heteroprotein complexes. Optimization of ET through the linkers between RC and other proteins

This research effort led to the discovery of the spontaneous formation of RC-Cytochrome complex between immobilized of electrode RC protein and Cytochrome c added to aqueous phase. Base on surface plasmon resonance band shift the RC-Cyt complex has protein ratio 1:1 and high binding constant similar to observed for these proteins in vivo. Discovered that incorporated cytochrome acts as a conductive wire increasing the efficiency of electron transfer between RC and electrode for up to 100 fold. We discovered that in addition to increasing conductivity due to the shortening of electron tunneling distance, cytochrome stabilizes RC protein orientation relative to the electrode preventing RC protein from tilting or laying on the electrode surface. (**JACS** 128:12044-12045, 2006)

Specific task 3. Optimization of ET at Qb-binding site (years 2-3)

1. Site-directed modification of RC herbicide binding domain according to the analyte structure.
2. Construction of a quinone-based linker for specific Qb binding
3. Modification of the quinone-based linker for optimization of conductive performance

The research in this direction led to the construction of a innovative type of molecular wires having quinone head at one end and a thiol or pyrene group at the other end connected by oligo(phenylenevinylene) forming electron delocalized bridge. The constructed molecular wires have increased electron transfer activity up to 1000 folds (depending on the pH) and can serve both for protein immobilization and wiring to the electrode (**Langmuir** 23:942-948, 2007, **Journal of Electroanalytical Chemistry** 606:33–38, 2007, **JACS,** 130:5578-5585, 2008).

Specific task 4. Construction of stable heteroprotein ET complex based on chimeric HSU (year 3)

1. Construction and analysis of ET in LMSU RC (RC with stripped HSU). Constructed LM protein complex and demonstrated increased stabilization of separated charges in it for more that 100 times.
2. Construction of a fusion chimera between H-subunit and another ET protein (to be determined). Chemically synthesized a polypeptide identical to the native α-helical N-

terminal end of H-subunit and demonstrated that the constructed polypeptide immediately and efficiently folds in solution in α-helix. Surface plasmon resonance experiments demonstrated that the helix plays crucial role in H-subunit binding to the rest of RC protein (LM-subunits) that beside possible applications for the formation of protein heterocomplexes hade the light of the origin of RC subunit components and mechanism of their assembly in vivo.

Specific task 5. Construction of RC-light-harvesting complexes (year 4)

1. Construction of RC-QD complexes at HSU. RC-QD complex was assembled using thioctic acid as charged QD coat allowing for efficient QD to RC binding. That eliminated the needs of using α-helix from HSU for QD to RC binding. The constructed complex demonstrated highly efficient transfer of light energy absorbed by QD specifically to RC primary donor. Demonstrated that the efficiency of energy transfer between QD and RC is controlled by the redox state of RC primary donor.
2. Assembly of RC-LH complexes on carbon surfaces. Site-directed modification of LH proteins for direct binding to carbon
3. Identification of components and principles of RC-LH association in vivo and construction (by SDM) of stable RC-LH complexes for in vitro performance
4. Site-directed modification of pigment binding domains for possible bacteriochlorophyll chromatic shifts
5. Site-directed modification of pigment binding domains for binding other porphyrins

After initial experiments with LH protein the work in this direction was shifted to the construction of innovative LH antenna for RC protein based on metallic nanoparticles. The initial theoretical estimations pointed out that the formation of surface plasmon polaritons can be much more efficient in concentration light energy at photosynthetic protein monolayers sitting on gold and silver nanoparticles and nanorods and also can transfer the energy for much longer distance than traditional Forster exciton transfer. While moving in this direction we have developed the strategy for the assembly of RC-nano-gold particles and perform initial experiments on the estimation of the efficiency of electron transfer in the constructed complexes.

Specific task 6. Assembly of stochastic RC-functionalized carbon electrode (year 5)

1. Construction of multi RC complexes on CNT. Identification of the possibility of RC functional interaction and cooperation at dense package.
2. Identification of potential binding domain for RC lateral association. Construction of stable RC –functionalized CNT with desired RC patterns {possibly involves lipid mono- or bilayers}
3. Construction of photoactivated semipermiable lipid membranes (RC+PORIN) at the top of CTN

We have constructed a new type of photoactive materials with photosynthetic RC proteins encapsulated inside CNT arrayed electrode. We have discovered that this encapsulation leads to substantial improvement of both electrode functional density and the rate of electron transfer

between RC and electrode. These results open the possibility to the construction of photovoltaic and photo sensing devices based on completely different principles compared to the traditional devices utilizing lamella architecture. (**Langmuir**, accepted 2008)

In addition to the completing the studies being in our initial plan and described above, several new directions were explored based on new discoveries and the results obtained in the course of our work. These include:

1. Theoretical analysis and the development of software for the identification of electron transfer pathways in the proteins and between the protein and inorganic electrode. This work led to the discovery of the important role of the electrode curvature on the rate of electron transfer allowing at optimal conditions for increasing of ET efficiency for several orders of magnitude.

2. Theoretical analysis and computer simulation of the possibility of using the surface plasmon polariton in noble metals for directed long-range energy transfer to RC monolayer.

3. The development of experimental approaches to the construction of a new type of single molecular switches with redox controlled changes of their conductivity. The changes in conductivity were show to be about 40 and are completely reversible (ACS Nano, accepted, 2008).

4. The developed experimental approaches to the estimation of electron transfer through a single RC protein. In addition to the clearly visible rectification effect on each single protein that itself is a big challenge, we have discovered conductance steps corresponding to its ET cofactors.

5. We have analyzed the energy transfer in the constructed RC-gold nanoparticles complexes using enhanced Raman scattering. These experiments proved the possibility of light energy concentration by metallic nanoparticles on RC, and showed the crucial role of the orientation of pigment dipole transition moment in this process.

Research Timeline

Specific task 1. Binding of RC to carbon NT

	6 mo	12 mo	18 mo	24 mo	year 3	year 4	year 5
RC cloning, expression and purification	▬						
Construction of site-specific linkers	▬	▬					
Demonstration of optical switching effect		▬					
mutants with different Cys locations			▬				
mobile carriers and phases	▬						

Specific task 2. Construction of a stable heteroprotein complex at the donor side of RC

	6 mo	12 mo	18 mo	24 mo	year 3	year 4	year 5
amino acids controlling RC-Cyt binding		▬					
Bifunctional linker for RC-protein binding			▬				
Conductive linker in heteroprotein complexes				▬			

Specific task 3. Optimization of ET at the Qb-binding site

	6 mo	12 mo	18 mo	24 mo	year 3	year 4	year 5
herbicide binding domain				▬			
Quinone-based linker head				▬			
Quinone-based linker tail					▬		

Specific task 4. Construction of stable heteroprotein ET complexes based on chimeric HSU

	6 mo	12 mo	18 mo	24 mo	year 3	year 4	year 5
LMSU RC				▬			
HSU chimera					▬		

Specific task 5. Construction of RC-light-harvesting complexes

	6 mo	12 mo	18 mo	24 mo	year 3	year 4	year 5
RC-QD					▬		
RC-LH association on carbon						▬	
Pigment-binding domains						▬	
RC-LH association in vivo							▬

Specific task 6. Assembly of stochastic RC-functionalized carbon electrode

	6 mo	12 mo	18 mo	24 mo	year 3	year 4	year 5
multi RC-CNT complexes						▬	▬
RC lateral association							▬

Summary of Mutations Put Into L-Subunit

Subunit	Sites* modified (original aa/substituted aa)	Clone (alt design)	RC	Storage Conditions	
				temperature	Rack/box
L	(Arg ^{7}/Cys ^{7})	R7	+	-70 NF	
L	(Arg ^{7}/Cys ^{7}) (Cys^{92}/Ala^{92})	dm-1	+	-70 NF	
L	(Arg ^{7}/Cys ^{7}) (Cys^{92}/Ala^{92}) (Glu ^{205}/Cys ^{205})	TM-1	+	-70 NF	
L	(Arg ^{7}/Cys ^{7}) (Cys^{92}/Ala^{92}) (Glu ^{205}/Cys ^{205}) HexHis 72 replacement (ALEYGL→HHHHHH)	TM.EH (TM.1HH)	-	-70 NF	
L	(Arg ^{7}/Cys ^{7}) (Cys^{92}/Ala^{92}) (Glu ^{205}/Cys ^{205}) HexHis 72 replacement (ALEYGL→HHHHHH) HexHis 277 replacement (NIPGGI→HHHHHH)	TM.2HH		-70 NF	
L	(Arg ^{7}/Cys ^{7}) (Cys^{92}/Ala^{92}) (Glu ^{205}/Cys ^{205}) HexHis 277 replacement (NIPGGI→HHHHHH)	TM.LH		-70 NF	

Key:

Sites*; depending on sequence used, positions vary. Figure below indicated residues changed.
RC+ ; cells make reaction center
RC- ; cells do not make reaction center
NF = Nikolai freezer in Room 216 chemistry

gi|132182|sp|P02954|RCEL_RHOSH Reaction center protein L chain (Photosynthetic reaction center L subunit)

```
  1  MALLSFERKY RVPGGTLVGG NLFDFWVGPF YVGFFGVATF FFAALGIILI

 51  AWSAVLQGTW NPQLISVYPP ALEYGLGGAP LAKGGLWQII TICATGAFVS

101  WALREVEICR KLGIGYHIPF AFAFAILAYL TLVLFRPVMM GAWGYAFPYG

151  IWTHLDWVSN TGYTYGNFHY NPAHMIAISF FFTNALALAL HGALVLSAAN

201  PEKGKEMRTP DHEDTFFRDL VGYSIGTLGI HRLGLLLSLS AVFFSALCMI

251  ITGTIWFDQW VDWWQWWVKL PWWANIPGGI NG
```

Individual residues changed in RED.

Site of hexahis replacements, shown below, in the fully substituted mutant:

1 MALLSFE C KY RVPGGTLVGG NLFDFWVGPF YVGFFGVATF FFAALGIILI

51 AWSAVLQGTW NPQLISVYPP HHHHHHH GGAP LAKGGLWQII TI A ATGAFVS

101 WALREVEICR KLGIGYHIPF AFAFAILAYL TLVLFRPVMM GAWGYAFPYG

151 IWTHLDWVSN TGYTYGNFHY NPAHMIAISF FFTNALALAL HGALVLSAAN

201 PEKGK C MRTP DHEDTFFRDL VGYSIGTLGI HRLGLLLSLS AVFFSALCMI

251 ITGTIWFDQW VDWWQWWVKL PWWA HHHHHHH NG

#	name	Δ L subunit	Δ M subunit	comment
	New Mutants			
1	L159 {N to C}	one change	none	new; designed new SDM primers
2	L159 L163 {N to C}, {T to C}	two changes	none	Modified the existing L163; plasmid is made designed new 159 SDM primer as it likely will not be same as for L159 alone, but may be influenced by preexisting L163
3	L256 or L258 {F to C}, {Q to C}	one change	none	258 alone; designed new SDM primers for 256
4	L281 {G to C)	C-end	none	new, easiest to do by re-amplification with reverse primer not a SDM reaction. designed new reamplification primer for 281
5	L72 {E to C}	one change	none	new; designed new SDM primers
6	M100 or M110 {E to C}, {K to C}	none	one change	we should have made SDM 100 as part of L163M100; designed new SDM primers for 110
7	M191 or M292 {L to C }, {D to C}	none	one change	we already had M191 alone; designed new SDM primers for M292

Explicit sequence modifications: L subunit

ALLSFERKYRVPGGTLVGGNLFDFWVGPFYVGFFGVATFFFAALGIILIAWSAVLQGTWNPQLI
SVYPPALEYGLGGAPLAKGGLWQIITICATGAFVSWALREVEICRKLGIGYHIPFAFAFAILAY
LTLVLFRPVMMGAWGYAFPYGIWTHLDWVSNTGY T163 YGNFHYNPAHMIAISFFFTNALALALH

GALVLSAANPEKGKEMRTPDHEDTFFRDLVGYSIGTLGIHRLGLLLSLSAVFFSALCMIITGTI
WFDQ²⁵⁸WVDWWQWWVKLPWWANIPGGI²⁷⁹NG

>**L72**
ALLSFERKYRVPGGTLVGGNLFDFWVGPFYVGFFGVATFFFAALGIILIAWSAVLQGTWNPQLI
SVYPPALEYGLGGAPLAKGGLWQIITICATGAFVSWALREVEICRKLGIGYHIPFAFAFAILAY
LTLVLFRPVMMGAWGYAFPYGIWTHLDWVSNTGYTYGNFHYNPAHMIAISFFFTNALALALHGA
LVLSAANPEKGKEMRTPDHEDTFFRDLVGYSIGTLGIHRLGLLLSLSAVFFSALCMIITGTIWF
DQWVDWWQWWVKLPWWANIPGGING

>**L159**
ALLSFERKYRVPGGTLVGGNLFDFWVGPFYVGFFGVATFFFAALGIILIAWSAVLQGTWNPQLI
SVYPPALEYGLGGAPLAKGGLWQIITICATGAFVSWALREVEICRKLGIGYHIPFAFAFAILAY
LTLVLFRPVMMGAWGYAFPYGIWTHLDWVSNTGYTYGNFHYNPAHMIAISFFFTNALALALHGA
LVLSAANPEKGKEMRTPDHEDTFFRDLVGYSIGTLGIHRLGLLLSLSAVFFSALCMIITGTIWF
DQWVDWWQWWVKLPWWANIPGGING

>**L159 or L163**
ALLSFERKYRVPGGTLVGGNLFDFWVGPFYVGFFGVATFFFAALGIILIAWSAVLQGTWNPQLI
SVYPPALEYGLGGAPLAKGGLWQIITICATGAFVSWALREVEICRKLGIGYHIPFAFAFAILAY
LTLVLFRPVMMGAWGYAFPYGIWTHLDWVSNTGYTYGNFHYNPAHMIAISFFFTNALALALHGA
LVLSAANPEKGKEMRTPDHEDTFFRDLVGYSIGTLGIHRLGLLLSLSAVFFSALCMIITGTIWF
DQWVDWWQWWVKLPWWANIPGGING

>**L256**
ALLSFERKYRVPGGTLVGGNLFDFWVGPFYVGFFGVATFFFAALGIILIAWSAVLQGTWNPQLI
SVYPPALEYGLGGAPLAKGGLWQIITICATGAFVSWALREVEICRKLGIGYHIPFAFAFAILAY
LTLVLFRPVMMGAWGYAFPYGIWTHLDWVSNTGYTYGNFHYNPAHMIAISFFFTNALALALHGA
LVLSAANPEKGKEMRTPDHEDTFFRDLVGYSIGTLGIHRLGLLLSLSAVFFSALCMIITGTIWF
DQWVDWWQWWVKLPWWANIPGGING

or

>**L258**
ALLSFERKYRVPGGTLVGGNLFDFWVGPFYVGFFGVATFFFAALGIILIAWSAVLQGTWNPQLI
SVYPPALEYGLGGAPLAKGGLWQIITICATGAFVSWALREVEICRKLGIGYHIPFAFAFAILAY
LTLVLFRPVMMGAWGYAFPYGIWTHLDWVSNTGYTYGNFHYNPAHMIAISFFFTNALALALHGA
LVLSAANPEKGKEMRTPDHEDTFFRDLVGYSIGTLGIHRLGLLLSLSAVFFSALCMIITGTIWF
DQWVDWWQWWVKLPWWANIPGGING

>L281
ALLSFERKYRVPGGTLVGGNLFDFWVGPFYVGFFGVATFFFAALGIILIAWSAVLQGTWNPQLI
SVYPPALEYGLGGAPLAKGGLWQIITICATGAFVSWALREVEICRKLGIGYHIPFAFAFAILAY
LTLVLFRPVMMGAWGYAFPYGIWTHLDWVSNTGYTYGNFHYNPAHMIAISFFFTNALALALHGA
LVLSAANPEKGKEMRTPDHEDTFFRDLVGYSIGTLGIHRLGLLLSLSAVFFSALCMIITGTIWF
DQWVDWWQWWVKLPWWANIPGGIN[G]

For the M-subunit

Explicit modifications in the M-subunit:

AEYQNIFSQVQVRGPADLGMTEDVNLANRSGVGPFSTLLGWFGNAQLGPIYLGSLGVLSLFSGLMWFFTI
GIWFWYQAGWNPAVFLRDLFFFSLEPPAP[100]YGLSFAAPLKEGGLWLIASFFMFVAVWSWWGRTYLRAQALGMGKH
TAWAFLSAIWLWMVLGFIRPILMGSWSEAVPYGIFSHLDWTNNFS[191]VHGNLFYNPFHGLSIAFLYGSALLFAMHG
ATILAVSRFGGERELEQIADRGTAAERAALFWRWTMGFNATMEGIHRWAIWMAVLVTLTGGIGILLSGTVVDNWYVW
GQNHGMAPLN

>M100
AEYQNIFSQVQVRGPADLGMTEDVNLANRSGVGPFSTLLGWFGNAQLGPIYLGSLGVLSLFSGLMWFFTI
GIWFWYQAGWNPAVFLRDLFFFSLEPPAP[E]YGLSFAAPLKEGGLWLIASFFMFVAVWSWWGRTYLRAQAL
GMGKHTAWAFLSAIWLWMVLGFIRPILMGSWSEAVPYGIFSHLDWTNNFSLVHGNLFYNPFHGLSIAFLY
GSALLFAMHGATILAVSRFGGERELEQIADRGTAAERAALFWRWTMGFNATMEGIHRWAIWMAVLVTLTG
GIGILLSGTVVDNWYVWGQNHGMAPLN

or

>M110
AEYQNIFSQVQVRGPADLGMTEDVNLANRSGVGPFSTLLGWFGNAQLGPIYLGSLGVLSLFSGLMWFFTI
GIWFWYQAGWNPAVFLRDLFFFSLEPPAPEYGLSFAAPL[K]EGGLWLIASFFMFVAVWSWWGRTYLRAQAL
GMGKHTAWAFLSAIWLWMVLGFIRPILMGSWSEAVPYGIFSHLDWTNNFSLVHGNLFYNPFHGLSIAFLY
GSALLFAMHGATILAVSRFGGERELEQIADRGTAAERAALFWRWTMGFNATMEGIHRWAIWMAVLVTLTG
GIGILLSGTVVDNWYVWGQNHGMAPLN

M191
AEYQNIFSQVQVRGPADLGMTEDVNLANRSGVGPFSTLLGWFGNAQLGPIYLGSLGVLSLFSGLMWFFTI
GIWFWYQAGWNPAVFLRDLFFFSLEPPAPEYGLSFAAPLKEGGLWLIASFFMFVAVWSWWGRTYLRAQAL
GMGKHTAWAFLSAIWLWMVLGFIRPILMGSWSEAVPYGIFSHLDWTNNFS[L]VHGNLFYNPFHGLSIAFLY
GSALLFAMHGATILAVSRFGGERELEQIADRGTAAERAALFWRWTMGFNATMEGIHRWAIWMAVLVTLTG
GIGILLSGTVVDNWYVWGQNHGMAPLN

or

M292
AEYQNIFSQVQVRGPADLGMTEDVNLANRSGVGPFSTLLGWFGNAQLGPIYLGSLGVLSLFSGLMWFFTI
GIWFWYQAGWNPAVFLRDLFFFSLEPPAPEYGLSFAAPLKEGGLWLIASFFMFVAVWSWWGRTYLRAQAL
GMGKHTAWAFLSAIWLWMVLGFIRPILMGSWSEAVPYGIFSHLDWTNNFSLVHGNLFYNPFHGLSIAFLY
GSALLFAMHGATILAVSRFGGERELEQIADRGTAAERAALFWRWTMGFNATMEGIHRWAIWMAVLVTLTG
GIGILLSGTVV[D]NWYVWGQNHGMAPLN

Flow-through Nanopore Bioreactor

Chuck Martin

We are collaborating with Prof. Rod Beresford and Prof. Joanne Yeh on a new type of flow through bioreactor. The bioreactor consists of conically shaped nanopores in a synthetic membrane, with conically shaped gold nanotubes deposited within the pores. A redox enzyme is immobilized on the inside walls of the gold nanotubes, and this enzyme ultimately will be hardwired to the gold. The conical shape is important because it is optimal for funneling the substrate molecules to the enzyme immobilized on the nanotube walls.

The key to this new redox bioreactor concept is the conical gold nanotube. We prepare these nanotubes via the well-known template-synthesis method by electroless deposition of gold within a correspondingly conically shaped pore. Such conically shaped pores are a relatively new concept in materials science, and we have pioneered their application in various fields of biomedical analysis and separations. We invested a considerable amount of effort during the course of the MURI effort to methods pf development for preparing such conically shaped pores. A key question underlying this research effort is — can the conically shaped nanopores that are the heart of this sensing/transducing strategy be prepared reproducibly? We have recently discovered that our track-etched conically shaped nanopores can be prepared with excellent reproducibility using a new two-step etching procedure. This new etching procedure is briefly reviewed here.

We obtain from the Gellsellschaft fur Schwerionenforschung (GSI) PET membranes (12 μm thick) that contain a single damage track through the membrane. Step 1 of the pore-etching process entails placing a solution that etches the damage track on one side of the membrane and a solution that neutralizes this etchant on the other side. For PET the etchant is NaOH and the etch-stop is formic acid. This "anisotropic" etch step yields a conically shaped pore with the base opening facing the etch solution and the tip opening facing the etch-stop solution. To determine when the etchant has broken through to the etch-stop solution, and a contiguous pore has been obtained, an electrode is placed in each solution, and a potential difference is applied across the membrane. Before breakthrough, the transmembrane current is zero, and breakthrough is signaled by a sudden rise in the current.

We previously showed that the diameter of the base opening could be controlled by varying the potential applied across the membrane during this first, anisotropic, etch step. Furthermore, good reproducibility in the base diameter is obtained. For example, an etch time of two hours was used in these studies, which yielded a base diameter of 520 ± 45 nm, as determined by scanning electron microscopy. However, the tip diameter obtained after this first etch step varied between 3 and 7 nm and could not be reproduced from etch to etch. We reasoned that this is an inherent feature of the anisotropic etch process. This is because the etch and etch-

stop solutions are mixing (and neutralizing each other) in the nascent tip, which makes it difficult to control the etch rate in this critically important region of the nanopore.

For this reason, after the first etch step, the membrane was etched again, but this time with the same NaOH etch solution on both sides of the membrane; hereafter called the "*isotropic etch.*" A transmembrane potential was applied during this isotropic etch step, and the ion current flowing through the nanopore was measured as a function of time during this etch. Our key innovation is that the second etch was stopped at a prescribed value of this nanopore ion current, rather than at some prescribed time after starting the etch. We adopted this approach because of the variability in tip diameter obtained after the first etch step. The consequence of this variability is that if we stopped the second etch at a prescribed time, we would obtain a corresponding variability in the tip diameters obtained after the second etch step. In contrast, the ion current flowing through the nanotube during the second etch uniquely defines the diameter of the tip opening. Hence, conceptually, we fix the base diameter in the first, anisotropic, etch step and then adjust the tip diameter in the second, isotropic, etch step.

The figure below shows the results obtained with this two-step etch method. This figure is a plot of the tip diameter obtained after the second etch vs. the nanopore ion current at which the etch was stopped. The error bars on the experimental data represent averages of tip diameters measured from at least three membranes prepared under identical conditions. We see that the reproducibility in the tip diameter is excellent, and as we have noted above, the reproducibility in the base diameter is also excellent. Furthermore, this figure shows that we can reproducibly make tips with diameters in the critically important range from 5 to 40 nm.
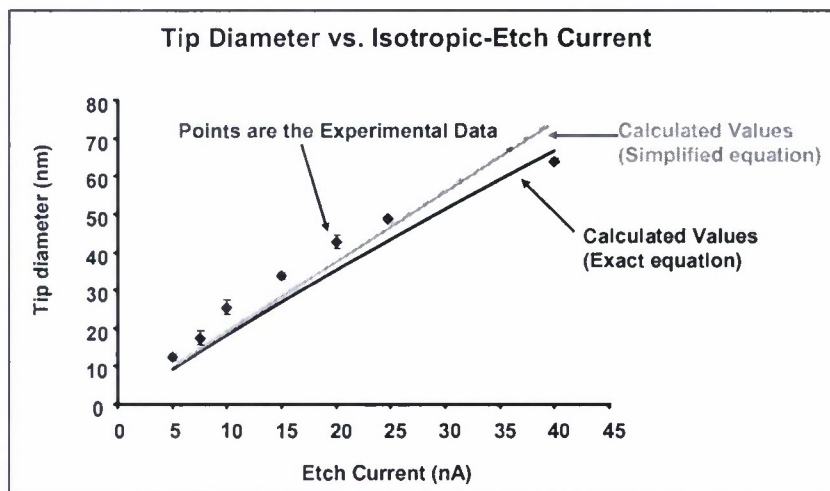
Finally, we have developed a simple theory that allows us to predict *a priori* and with no adjustable parameters what the tip diameter should be, given the etch parameters. The solid curves show the results of both a simplified and the exact version of this theory. Good agreement between the experimental and theoretically predicted values is obtained, we also understand why the theory, in general, slightly underestimates the experimental tip diameter. In short, this is an experimental problem (not a theoretical one) which results because we do not quench the etch quickly enough, so the tips end up a little bit bigger than the theory predicts.

In conclusion, we have taken a major step in transitioning our conical nanopore transducers from an interesting laboratory experiment to a practical real-world technology.

For the bioreactor side of the project we are currently investigating two enzymes — an NADH peroxidase that we have obtained from Prof. Yeh, and the gold standard of redox enzymes, glucose oxidase (GOD). Because there is so much known about GOD and because it is an especially hardy redox enzyme, we have begun our studies with GOD. We have completed a series of preliminary studies in collaboration with Prof. Beresford's group. We successfully immobilized the GOD to the conical gold nanotubes using well-known glutaralydehyde-based chemistry. We investigated the electrochemistry of the attached GOD in the absence and presence of flow of glucose-containing solution through the gold nanotubes. These preliminary studies suggest that this system is electrochemically active, but more work remains to be done. We are currently investigating the effect of flow rate of solution through the conical nanotubes on the efficiency of glucose oxidatio n.

Microfluidic Electrochemical Detectors:
Nanoscale Integration and Information Extraction

Rod Beresford

Progress is again being reported along three related directions: process development and chip characterization for the integration of nanoelectrodes in a planar fabrication sequence (Flow Chip III), demonstration of conical nanotubes as efficient sensors in a flow-through configuration (Flow Chip IV), and system integration of micro- and nano-electrochemical sensing with hardware and software for information extraction (Flow Chip V). The Beresford group created planar fabrication modules that can be employed to add precisely calibrated electrochemical sensing with nanoelectrodes in microfluidic channels on top of an existing integrated circuit. Flow Chip II (prior work) is a well-calibrated microscopic three-electrode electrochemical sensor integrated with a polydimethylsiloxane (PDMS) microchannel to form a rapid-prototype chip technology for MURI team linker-system characterization *in situ*. This chip was the basis for other developments as reported here.

$$Ru(NH_3)_6^{3+} + e^- \rightleftharpoons Ru(NH_3)_6^{2+}$$
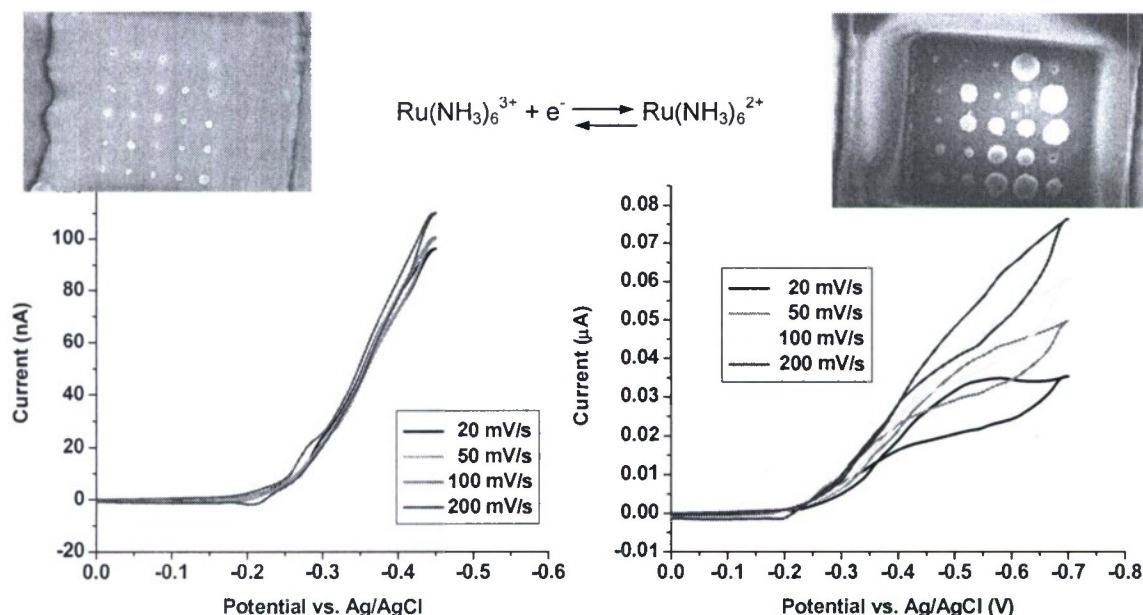
Figure 1. Flow Chip III comprises Au nanodot integration with the 3-electrode sensing configuration of Flow Chip II. Focused-ion-beam fabricated pores are filled with Au by electrodeposition. The normalized current $I/C\sqrt{v}$ can serve as a yardstick to confirm that the observed current correlates to the observed geometric area of the ensemble (and not to leakage current paths associated with the underlying wiring). The value of $I/C\sqrt{v}$ is found from a microelectrode experiment (not shown), where the leakage problem is not a factor. On the left, the observed geometric area of the Au nanodot ensemble is 2.73×10^{-8} cm^2, but the electrical surface area is almost 50 times larger, indicating leakage current paths. On the right, after process developments to improve the insulator layer, the electrical surface area is less than 4 times greater than the geometric area, which is a more typical factor that can be accounted for by surface roughness of the nanoelectrodes.

In Flow Chip III (Fig. 1), Au nanodot / nanowire structures are integrated with the three-electrode system of Flow Chip II. The resulting nanodot arrays have been characterized with redox standard species, confirming successful electrical design, which is complicated by the requirement of limiting the parasitic capacitive coupling from the underlying wiring to the solution. These arrays were tested in collaboration with Prof. Joanne Yeh, detecting PSA (prostate specific antigen) at low concentration (below nanomolar), Fig. 2.
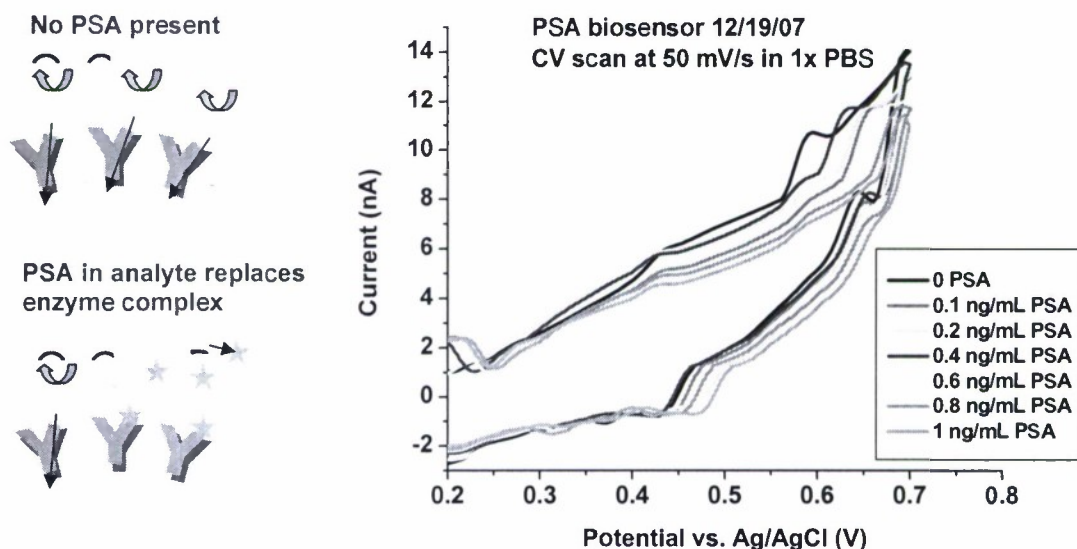
Figure 2. Schematics at left show the operating principle of the PSA detector, in which a PSA+GOx complex initially is bound to the linker complex (PSA antibody), which attaches to the Au nanodots. PSA in the analyte then displaces the PSA+GOx complex, reducing the electron transfers to the nanodots. The CV results show reduced current with added PSA, as intended. PSA concentrations as low as 0.01 ng/mL were detected with this method.

One of the inherent limitations of the flow-chip geometry adopted here is that most of the mass involved in the laminar flow in a microfluidic channel of some 10 – 15 µm height is never in contact with the nanostructure probes that are a fraction of a micrometer in height and attached to one boundary of the channel. A target molecule that is present at picomolar concentration in a channel of total volume 100 nanoliters is present at the level of 60,000 copies only. To sense these low concentrations, it will be extremely useful to have a flow-through configuration, in which most of the fluid volume is forced to interact with the probes. In collaboration with the Martin and Xu groups, such a flow-through configuration has been achieved (Flow Chip IV). As shown in Fig. 3, a PET membrane containing conical nanopores with tip openings down to 70 nm is captured between two fluidic channels, one in glass and one in PDMS. Electrical contact is formed to the Au plating on the membrane in the same operation. For comparison, working electrodes similar to those of Flow Chip II are included as controls.

Base opening 1 μm

Tip opening 70 nm

PET   Au 20 nm   S-(CH₂)₂-N=CH-(CH₂)₃-CH=N-(GOx)

MPE-Glutaraldehyde

9/27/07
GOx no flow in PBS pH 7.4

GOx PBS pH 7.4 flow rate 0.5 uL/min

Current (uA)

Potential vs. Ag/AgCl (V)

Potential vs. Ag/AgCl (V)

— 20 mV/s
— 50 mV/s
100 mV/s
200 mV/s
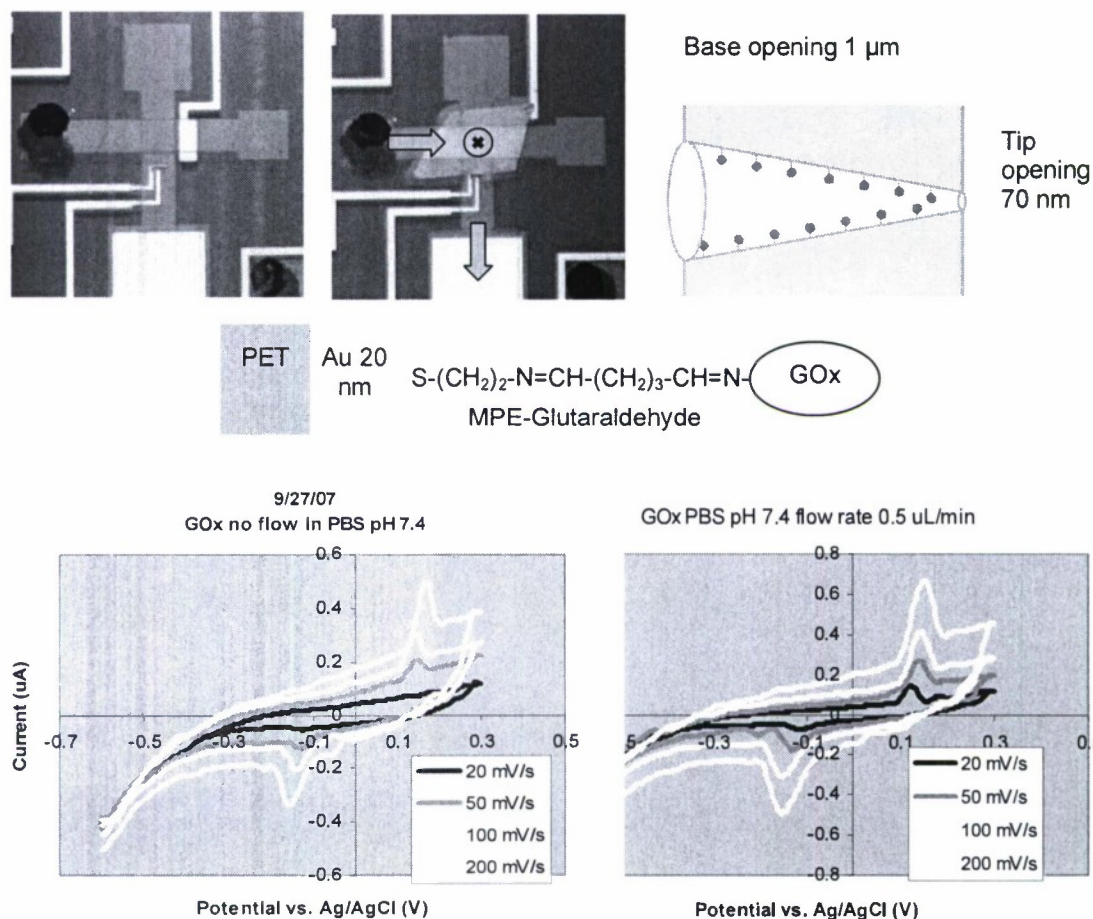
Figure 3. Flow Chip IV comprises integration of conical gold nanotubes formed in a track-etched PET membrane with a planar electrode array similar to Flow Chip II. Photos at upper left show crossed channels without and with the captured membrane; superimposed arrows show the flow directions. CV data clearly show oxidation and reduction of a species at the membrane electrode (no such features are present at the control electrodes). However, it is not known yet whether the peaks relate to the thiol of the linker (possible, though the voltage is too negative compared with literature values for this reaction) or the GOx (which generally is not susceptible to direct electrochemistry, but may become active in nanostructured environments). Further testing is required to quantify glucose oxidation efficiency resulting from this configuration.

A transimpedance amplifier configuration with a small integration capacitor is widely used for low-noise sensing of sub-picoampere currents, including commercially for reading out photogenerated charges in focal-plane arrays. Off-the-shelf integrated circuits are available that implement the read-out function separately from the sensor function. These read-out integrated circuits (ROICs) target infrared sensors, which employ a variety of exotic (non-silicon) detector materials. However, there is no reason from the signal processing perspective that one cannot target as the detector material an aqueous electrolyte instead of a semiconductor junction. In order to resolve questions about the ROIC performance in this environment, and to establish a

prototype system design, we are implementing a redox-reaction imaging system based on a commercial ROIC. An initial system design has been completed, including all digital and analog elements required to interface the ROIC to a computer and to the electrochemical experiment (Fig. 4). Construction of the prototype is continuing. Although this approach was not specifically envisioned in the MURI proposal, it represents a fascinating and potentially productive new research direction enabled by the MURI research. A follow-on proposal in this area was submitted to NSF during the past year. If further funding can be secured, this work promises new capabilities for time-resolved imaging of chemically heterogeneous environments, including cells and their colonies.
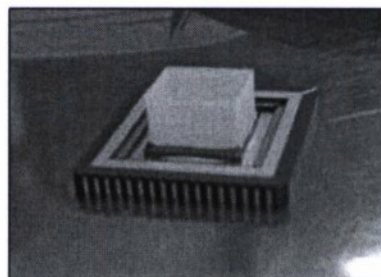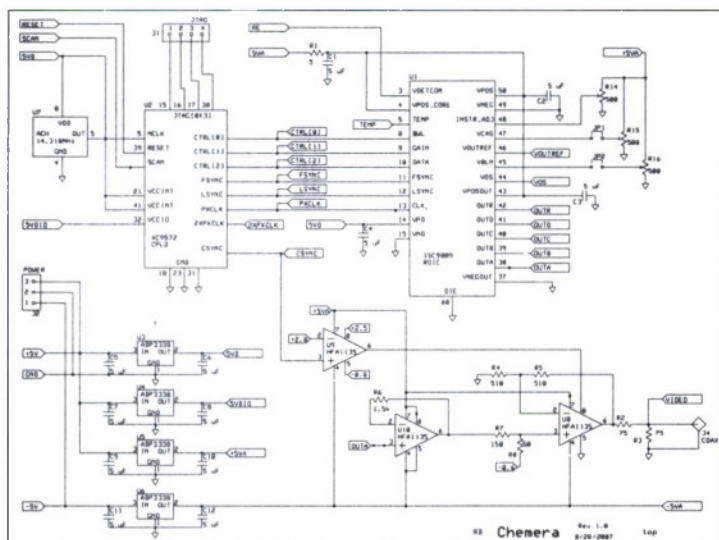

Figure 4. Flow Chip V comprises a system integration demonstration of hardware and software for information extraction. A commercial ROIC provides the pixel array of working electrodes (photo); additional electronics design is exploited for control and read-out (schematic at left).

2008 MURI Cumulative Performance Metrics (Final)

1. Publications 122

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121. Xia, T., Kovochich, M., Liong, M., Lutz, M., Shi, H., Yeh, J.I., Zink, J.I., and Nel, A.E. (2008). Comparison of the Mechanism of Toxicity of Zinc Oxide and Cerium Oxide Nanoparticles Based on Cellular Uptake Mechanisms and the Hierarchical Oxidative Stress Paradigm. *Submitted.*

122. Yeh, J.I., Chinte, U., and Du, S. (2008). Structure of glycerol-3-phosphate dehydrogenase, an essential monotopic membrane enzyme involved in respiration and metabolism. *PNAS USA* **105**(9), 3280-3285.

2. Conference papers

123. J.M. Xu, (Invited), "Interfacing Biomolecules with Nanoelectronics", Gordon Conference on Computational Chemistry, July 4-9, 2004, New Hampshire.

124. J.M. Xu, (Invited), "Bio-nanoelectronics", Seoul National University, Seoul, Korea, August 26, 2004

125. Control and System Integration of Micro and Nano-Scale Systems, Report from the National Science Foundation workshop, Washington, DC, March 29–30, 2004 p.87

126. Strategic Research to Enable NASA's Exploration Missions Conference, Cleveland, Ohio, June 22-23, 2004 p.103

127. S.O. Kelley et al., Ultrasensitive Biosensing using DNA-Modified Nanostructures," *SANKEN Research Symposium*, Osaka, Japan, Feb. 2005.

128. S.O. Kelley et al., "Chemical Approaches to DNA Electronics and Photonics," *DARPA Workshop on DNA Photonics*, Hawaii, April 2005.

129. S.O. Kelley et al., "Ultrasensitive Biosensing using DNA-Modified Nanostructures," *Materials Research Society dinner speaker*, Northeastern University, Boston, April 2005.

130. S.O. Kelley et al., "Ultrasensitive Biosensing using DNA-Modified Nanostructures" *Center for Nanomaterials Symposium*, Argonne National Laboratories, May 2005.

131. SPIE 2004: Contarino, MR.; Kamat, V; Keough, E; Babu, NS.; Sergi, M; Ishino, T; Papazoglou, E; Chaiken, IM.; Tyagi, S; Pourrezaei, K. "Bio-nano-optics for cellular investigations", in Medical and Biomedical Sensor Technology II, Proc. SPIE 5588, 31-40 (2004).

132. Moore, M.H., Trammell, S.A., Pollack, S.K., Lebedev, N., Kushmerick, J.G.: Optimizing charge transfer between photosynthetic proteins and electrode surfaces. - Abstracts of Papers of the American Chemical Society 229: U664-U664 214-COLL Part 1; 13 March 2005

133. Spano, A., Lebedev, N.: Construction of RC protein mutants with improved electron-transfer to inorganic electrode. – Gordon Research Conference in Photosynthesis, Smithfield, RI; 3-8 July 2005

134. Lebedev, N., Trammell, S.A., Spano, A., Griva, I.: Photoinduced electron transfer between photosynthetic proteins and electrodes. – Organic Microelectronic Workshop, Newport, RI; 11-13 July 2005

135. Lebedev, N., Trammell, S.A., Spano, A., Griva, I.,Goodman, B.: Photo-regulated electron transfer at bio-inorganic interfaces. – International Conference on Bio-Nano-Information Fusion, Marina-del-Rey, CA; 20-22 July 2005

136. Lebedev, N., Trammell, S.A., Spano, A., Griva, I.: Photoactive Protein-Functionalized Surfaces. –Workshop on Multifunctional Materials, Keystone, CO; 21-26 August 2005

137. Trammell, S.A., Spano, A., Lebedev, N.: Photoelectrochemical characterization of photosynthetic reaction centers on Ni-NTA self-assembled monolayers of different lengths on gold. – Second World Congress on Synthetic Receptors, Salzburg, AU; 7-9 September 2005

138. S. Zhang, *French Nanotechnology Conference*, Dec. 2004, Paris, France

139. S. Zhang, 2005 Spring MRS meeting, April 2005, San Francisco,

140. S. Zhang, Spring ACS meeting, March 2005, San Diego,

141. S. Zhang, *VIth European Protein Society Symposium*, April 2005, Barcelona, Spain.

142. S. Zhang, *Protein Design*, Jerusalem, Israel, May 2005

143. S. Zhang, *Nobel Symposium: Molecular Oncology: From Bench Side to Bedside*, June 17-19, 2005 Stockholm, Sweden

144. S. Zhang, *First Joint Nobel Symposia: Energy in Cosmos, Molecules and Life*, June 19-23, 2005, Stockholm, Sweden

145. S. Zhang, 4th Crete Multidisciplinary Workshop, June 25-28, 2005, Crete, Greece

146. S. Zhang, *European Self-assembled Fiber Network*, June 29-30, 2005, Crete, Greece

147. S. Zhang, Sichuan University Summer School, Chengdu, China, July 2005

148. S. Zhang, Summer ACS meeting, August 2005, Washington, D.C.

149. N. Triroj and R. Beresford, "Microfluidic Integration of Ultramicroelectrode Cells for Direct Electronic Detection of Biomolecular Signals," Mat. Res. Soc. Spring Meeting, March 2005, San Francisco, CA.

150. Yeh, J.I. and Nel, A. "Integration of High-Resolution Biomolecular Structures and Function in Nanomedicine and Nanosensor Applications" Proceedings, International Congress of Nanotechnology, San Francisco, California (11/05).
151. Whithey, G., Lazarek, A., Tzolov, M.B., Yin, A., Aich, P., Yeh, J.I., and Xu, J. "Enhanced redox enzyme activity using highly ordered carbon nanotube electrodes". International Conference on Bio-Nano-Informatics (BNI) Fusion; UCLA (07/05).
152. N. Mao and Yeh, J.I. "Membrane Protein Prediction of *M.tb* using SVM Algorithm". IEEE Trans. on Signal Transduction, *in press*. IEEE Genomic Signal Processing and Statistics Conference; Newport, RI (05/05).
153. Yeh, J.I. "Manual Nanocrystallization and Enhancing Diffraction *in-situ* by Annealing" Practical Aspects of Structure Determination Using Synchrotron Radiation; Argonne National Labs, SER-CAT Symposium Proceedings (03/05).
154. Yeh, J.I. "*In-situ* Detection and Quantification of Reactive Oxygen Species using Nanobiosensors" Proceedings, International Congress of Nanotechnology, San Francisco, California (01/05).
155. N. Kouklin, W. Kim, J.M. Xu, "Functionalization Enhancement of Carbon Nanotubes for Bio-Assays", 206th Meeting of the Electrochemical Society, Honolulu, HI, October 3-8, 2004
156. A. Yin, L. Guo, K. Hanson, M. Tzolov, "Integration of Highly Ordered Carbon Nanotube Arrays onto Silicon Substrates", 206th Meeting of the Electrochemical Society, Honolulu, HI, October 3-8, 2004.
157. R.Beresford, J.Liang, H.Luo and J.M.Xu "Growth Pathway for Highly-Ordered Quantum-Dot Arrays", NAMBE 2004 - 22nd North American MBE conference – Banff, Alberta, Canada, Oct. 11-14, 2004
158. Tzolov, Marian; Kuo, Tend-Fang; Straus, Daniel; Yin, Aijun; Xu, Jimmy, "Carbon Nanotube / Silicon Heterostructures: A Pathway to Integration for Electronics and Sensing Applications", MRS 2004, Boston, November, 2004
159. Aijun Yin, Karl Hanson, Hope Chik, and Jimmy Xu, "Controlled growth and Postgrowth Processing of carbon nanotube arrays", MRS 2004, Boston, November 29- December 3, 2004.
160. R. Gasparac, B. J. Taft, A. D. Lazareck, J. M. Xu, and S. O. Kelley, "Electrochemical DNA detection at novel nanoelectrodes.", American chemical society national meeting 2004, Philadelphia, PA.
161. Aijun Yin, Karl Hanson, Hope Chik, Jimmy Xu, "Controlled Growth and Post-growth Processing of Carbon Nanotube Arrays", MRS Spring Meeting, San Francisco, Symposium U, pp.491, 2005.
162. W Guo, R Guico, R Beresford, J.M. Xu, "Growth of Highly Ordered InAs/GaAs Quantum Dots on Nonlithographically Patterned Substrates by Molecular Beam Epitaxy", 16th American Conference on Crystal Growth and Epitaxy, ACCGE-16, Montana, July 10-15, 2005.
163. A. Yin, P. Kossyrev, S. Cloutier, R. Guico, J. Kim and J.M. Xu, "Nonlithographic Fabrication of Nanostructured Arrays Using Anodic Aluminum Oxide Films Containing Highly Ordered Arrays of Pores of 10 to 50nm", Symposium "Sensors Based on Nanotechnology II", of the 208th Meeting of The Electrochemical Society, Los Angeles, California October 16 - 21, 2005.

164. Mark Contarino, Adam Lazareck, Mauro Sergi, Jimmy Xu, Irwin Chaiken, "Metal binding, self-assembling peptide linkers for carbon nanotube biosensor interfaces" Affinity 2005, Philadelphia, August, 2005.

165. 19th American Peptide Symposium. Boston: Cocklin S, Zurawski J, Cocklin SL, Sodroski J and Chaiken I. CCR5 Conformation is Intimately Associated with its Lipid Environment. August 2004.

166. European Protein Society, Barcelona, Spain, Apr/May 2005: Cocklin S, Weeks S, Robertson N, Young E, Seal S, Zhang M, Loll P, Saunders A, Rest R, and Chaiken I. Real Time Characterization of the Membrane-binding and Insertion Properties of the Cholesterol-Dependent Cytolysin anthrolysin O from Bacillus anthracis.

167. "Optimization of a redox protein-carbon nanotube conjugate biosensor by site-selective binding" Withey, G.D., Lazareck, A.D., Tzolov, M.B, Yin, A., Aich, P., Yeh, J.I. and Xu, J.M. Device Research Conference, UCSB, June 2005.

168. "Enhanced redox enzyme activity using highly ordered carbon nanotube electrodes".Withey, G., Lazareck, A., Tzolov, M.B., Yin, A., Aich, P., Yeh, J.I., and Xu, J. International Conference on Bio-Nano-Informatics (BNI) Fusion; UCLA, July 2005.

169. "Ultrasensitive Electrochemical Biosensing with Nanowire Electrodes", S.O. Kelley, *Bioanalytical Sensors Gordon Conference*, Ventura, CA, Feb. 2006.

170. Nikolai Lebedev, Scott Trammell, Stanislav Tsoi , Anthony Spano, Benjamin Goodman, Marc Baldo, Jonathan Mapel, Igor Griva: Construction of protein-based high power density photo-voltaic devices. - BioMolecular Motors PI Meeting, Arlington, VA, 7-8 February 2006

171. Igor Griva and Nikolai Lebedev: Computational analysis and simulation of Electron Transfer in Proteins. - BioMolecular Motors PI Meeting, Arlington, VA, 7-8 February 2006

172. Nikolai Lebedev: Towards construction of Bio-Inspired Integrated, Multifunctional, Autonomous, Self-Assembling Nanodevices and Systems. – DARPA Workshop on Nano-Enabled Devices and Systems for Defense Applications, Colorado Springs, CO, 11-12 April, 2006

173. Nikolai Levedev: Light Energy and Signal Transduction at Bio-Inorganic Interfaces. – NanoMaterials for Defense Applications Symposium, Virginia Beach, VA, 1-4 May, 2006

174. Nikolai Lebedev, Scott Trammell, Igor Griva, Anthony Spano, Martin Moore, Stanislav Tsoi, Joel Schnur: Light energy conversion in photosynthetic reaction center proteins at inorganic electrodes. SPIE Optics & Photonics Annual Meeting, San Diego, CA, 13-17 August, 2006

175. Yeh, J.I., Calorimetric Approaches to Characterizing Effects of Additives on Protein Crystallization" Proceedings International Conference on Crystallization of Biological Macromolecules. Quebec City, Canada.

176. Napat Triroj, Wei Guo, Newton Ikhariale, David Meisner, and Roderic Beresford, Microfluidic Nanoelectrode Cell Array for Direct Electrochemical Detection of Biomolecules, Gordon Conference on MEMS Technology and Biomedical Applications, New London, CT, July 2006.

177. Nano-Engineered Crystalline Silicon for Enhanced Photoluminescence and 1.28µm Laser Action," S.G. Cloutier, C.H. Hsu, P. Kossyrev, E. Rotem, J. Shainline, and J.M. Xu, Photonics West Conference, January 2006.

178. Optical Characterization of Highly Ordered InAs/GaAs Quantum Dots on Non-lithographically Patterned Substrates, R.S. Guico, M. Tzolov, W. Guo, S.G. Cloutier, R.

Beresford, and J.M. Xu, North American Molecular Beam Epitaxy Conference, Durham, NC, 2006.

179. Kinetic Monte Carlo Simulation of Pattern-Driven InAs Quantum Dot Growth, W. Guo, R.S. Guico, J.M. Xu, and R. Beresford, orth American Molecular Beam Epitaxy Conference, Durham, NC, 2006.

180. Pattern-Driven Growth of InGaAs/GaAs Nano-dots by Atomic Hydrogen-Assisted Molecular Beam Epitaxy, W. Guo, J. Shainline, A.J. Yin, J.M. Xu, and R. Beresford, International Conference on Crystal Growth, Park City, UT, 2007.

181. Nikolai Lebedev, Scott Trammell, Igor Griva, Anthony Spano, Stanislav Tsoi, Joel Schnur: New Bio-Inorganic Photo-Electronic Devices Based on Photosynthetic Proteins. – Nano Materials for Defense Applications Symposium, San Diego, CA, April 23-26, 2007

182. Nikolai Lebedev, Scott Trammell, Igor Griva, Anthony Spano, Stanislav Tsoi, Charles Dulcey, Joel Schnur: Light Energy Conversion by Photosynthetic Proteins at Inorganic Electrodes. – NanoElectronic Devices for Defense and Security Conference. Arlington, VA, June 18-21, 2007.

183. Nikolai Lebedev, Scott Trammell, Igor Griva, Anthony Spano, Stanislav Tsoi, Charles Dulcey, Joel Schnur: Light Energy Conversion by Photosynthetic Proteins at Inorganic Electrodes. - 13th International Conference on Unconventional Photoactive Systems, Chicago, IL, August 5-9, 2007.

184. Scott Trammell, Daniel A. Lowy, Dwight S. Seferos, Martin Moore, Guillermo C. Bazan, Nikolai Lebedev: Rapid proton-coupled electron-transfer of hydroquinone through de-localized bridges. – ACS Annual Meeting Boston, MA, August 20-24, 2007.

185. Nikolai Lebedev, Scott Trammell, Igor Griva, Anthony Spano, Stanislav Tsoi, Joel Schnur: Light energy conversion by photosynthetic reaction center proteins at inorganic electrodes. - SPIE Organic Photovoltaics VIII Conference August 27-31, 2007.

186. Anthony J. Spano, Frank S. Chen, Kiev Blasier, Benjamin Goodman, Martha N. Simon, Joseph S. Wall, John J. Correia, William W. Newcomb, Jay C. Brown, Joel M. Schnur and Nikolai Lebedev: Gene Transfer Agent, a New molecular NanoScaffold for targetable Draug Delivery. – American Society for Cell Biology Annual Meeting, Washington, DC, December 1-5, 2007.

187. S.O. Kelley, "Nanoscale Electrode Platforms for Ultrasensitive Biosensing" *Pittcon*, Chicago, IL, Feb, 2007.

188. Jimmy Xu, "Think big, act small – at the interface between nanoelectronics and biolmolecules", Guest Speaker, annual Science Convocation at Lock Haven University of Pennsylvania, Oct. 12th, 2006.

189. Jimmy Xu, "Bio-nanoelectronics", short course, IEEE Nano Materials, Devices, and Circuits Meeting, Korea, Oct. 21-26, 2006

190. Jimmy Xu, "Bio-nanoelectronicsL extending information technology to acquiring info and executing on info", Tufts University, Department of Electrical Engineering, Oct 31, 2006

191. Gary Withey, Jimmy Xu, "Protein-Nanoelectronic transduction", Intl Nano Giga Conference, Tempe, Arizon, March 9, 2007

192. Gary Withey, Jin Ho Kim, Jimmy Xu, "Scalable DNA-programmed Self-Assembly of Enzyme-Nanoelectronic Transducers", DoD NanoMaterials for Defense Applications Symposium, April 23-26, San Diego, 2007

193. Gary Withey, Jin Ho Kim, Jimmy Xu, DNA-programmed, scalable, renewable array of redox protein – nanoelectronic transducers, Engineering Foundation Conference on

Nanoscience & Nanotechnology for Biological/Biomedical/Chemical Sensing City University of Hong Kong, Hong Kong, June 8-11, 2007.

194. Jefferey Shainline, Efi Rotem (Xu group),"Directly Pumped Silicon Lasing", CLEO 2007, Session on "Optical Materials, Fabrication and Characterization", May 7-11, 2007, Baltimore, MD.

195. Jimmy Xu, "Arrayed carbon nanotube-silicon heterojunciton: a new IV-IV semiconductor system and uncooled IR sensing ", 2007 Workshop on Compound Semiconductors and ICs, Venice, Italy, May 20-23, 2007.

196. Efi Rotem, Jeffrey Shainline, Jimmy Xu, "Stimulated emission and emission efficiency enhancement in nanopatterned silicon", SPIE Optics East, Sept. 9-12 2007, Boston

197. Jimmy Xu, "NanoEngineered Silicon Laser-filling the void of silicon photonics", the 6th "Annual NanoMaterials for Defense Applications Conference", April 21-24, 2008, Washington D.C.

198. Jimmy Xu, "Bio-Nanoelectronics: Direct Nanoscale Conversion of Biomolecule Signals and Structures", Keynote, the 7th US-Korean Workshop on anomaterials and the 5th US-Korea Workshop on Nanoelectronics, 24-25 April 2008, Arlington, VA.

199. "Bio-Nanoelecronics", Jimmy Xu, IMNI Symposium, May 5-7, 2008, Brown University.

200. Gary Withey, Jin Ho Kim, Jimmy Xu, "DNA-programmed protein-nanoelectronic transducer array", Bio-Sensing symposium, paper 7035-21, SPIE Optics & Photonics Meeting, August 10-14, 2008 in San Diego, CA.

201. J.I. Yeh, "Structure of Glycerol-3-Phosphate Dehydrogenase, an Essential Integral Membrane Enzyme Involved in Modulation of Respiration, Energy Status, and Phospholipid Biosynthesis" (November 3, 2007). Speaker. International Conference of the Crystallographic Association (Taipei, Taiwan).

202. J.I. Yeh, "Coordinated Nanobiosensors for Enhanced Detection: integration of three dimensional structures to toxicological applications" (October 4, 2007). Speaker. Nanotechnology TX 2007 Conference, Dallas, Texas.

203. J.I. Yeh, "Coordinated Nanobiosensors for Enhanced Detection: integration of three dimensional structures to toxicological applications" (June 27, 2007). Speaker. International Cancer Nanotechnology 2007 Conference, Paris, France.

204. S. Tsoi; I. Griva; S.A. Trammell; A.S. Blum; J. Schnur; N. Lebedev: Molecular Conductance Switching Via Controlled Alteration of Electron Delocalization: Quinone-Modified Oligo(phenylenevinylene), - 2008 International Conference on Nanoscience + Technology, Keystone, CO, July 20-25, 2008

205. Frank Chen, Anthony Spano, Benjamin E. Goodman, Kiev R. Blasier, Agnes Sabat, Erin Jeffery, Andrew Norris, Jeffrey Shabanowitz, Donald F. Hunt, and Nikolai Lebedev: Identification of the Structural and Regulatory Proteins of *Rhodobacter capsulatus* Gene Transfer Agent. - Gordon Research Conference Photosynthesis, South Hadley, MA ,June 22-27, 2008.

206. Stanislav Tsoi, Igor Griva, Scott A. Trammell, Amy S. Blum, Anthony Spano, , Joel Schnur, and Nikolai Lebedev: Towards Hybrid Bio-Inorganic Photovoltaics Based On Photosynthetic Reaction Center Proteins. – Gordon Research Conference Photosynthesis, South Hadley, MA ,June 22-27, 2008 (invited talk).

207. Nikolai Lebedev, Scott A. Trammell, Stanislav Tsoi, Anthony Spano, Jin Ho Kim, Jimmy Xu, Mark E. Twigg, Charles Dulcey, and Joel Schnur: Construction of novel multifunctional photo-electronic material by encapsulation of photosynthetic reaction

center protein in carbon nanotube electrode/arrays. - NanoMaterials for Defence and Security Conference, Arlington, VA, April, 21-23, 2008.

208. I. Griva, J. Schnur, and <u>N. Lebedev</u>: 3D simulation of light propagation and enhancement in arrays of cylindrical nanowells. – NanoMaterials for Defence and Security Conference, Arlington, VA, April, 21-23, 2008

209. C.R. Martin "Counting Molecules with Nanotubes - A New Paradigm In Biosensor Design," (Invited Lecture) Pittcon 2008, New Orleans, LA, March 4, 2008.

210. J.I. Yeh, "Structure of Glycerol-3-Phosphate Dehydrogenase, an Essential Integral Membrane Enzyme Involved in Modulation of Respiration, Energy Status, and Phospholipid Biosynthesis" (May 30-June 3, 2009). Invited Speaker. Membrane Biology Frontiers: Dynamics, Energy, Structure, and Technology, Mykonos, Greece.

211. J.I. Yeh, "Diffraction and Symmetry in Macromolecular Crystallography". (October 29, 2008). Invited Speaker. 66th Annual Pittsburgh Diffraction Conference, Pittsburgh, PA.

212. J.I. Yeh, "Ultrasensitive Biosensors to Detect Clinically Relevant Molecular Biomarkers" (October 2-3, 2008). Invited Speaker. (October 2-3, 2008). University of Pittsburgh, Science 2008 Conference, Pittsburgh, PA.

213. J.I. Yeh, "Ultrasensitive Biosensors to Detect Clinically Relevant Molecular Biomarkers" (Sept. 19-20, 2008). Invited Speaker. Fifteenth Annual Optic Nerve Rescue and Restoration Think Tank, New York, New York.

214. J.I. Yeh, "Structures of Octameric Hemoglobin: Correlation of Oligomeric States to Oxygen Binding Properties and Clinical Applications" (September 11, 2008). Invited Speaker. INSERM U779, Hopital de Bicetre, Paris, France.

215. J.I. Yeh, "Structure of Glycerol-3-Phosphate Dehydrogenase, an Essential Integral Membrane Enzyme Involved in Modulation of Respiration, Energy Status, and Phospholipid Biosynthesis" (August 23-29, 2008). International Conference of the Crystallographic Association, Osaka, Japan.

3. Transitions and consultations 151

1. Continuous info exchange with NRL (via the group of Dr. Nikolai Lebedev); and with Dr. Nalin Kumar, President, UHV Technologies, Inc., NJ.
2. Joint development of nanotube sensors with Radiance Corp., AL
3. Collaboration with and technology transition to Marine Biology Lab, Woods Hole, MA
4. Collaboration with and technology transition to Liver Research Center, RI.
5. Collaboration with Oak Ridge National Lab, TN
6. Collaboration with Aids Research Center, RI.
7. Joint development agreement Textron and Motorola for nanotube array based electrochemical and nanoelectronic electrodes (Xu)
8. Molecular Imaging Ltd.. – Co-development of novel Atomic Force Microscopy linking protocols for molecular specific binding, recognition, and probing. MI contributed $20K hardware (Xu).
9. Carbon nanotube arrays for bio-fuel cells to Naval Research Laboratory
10. Nano-pillar arrays for radiation sensing to AFRL – Kirtland.
11. Dr. Dave Cardimona and Dr. Dan Huang, AFRL – Kirtland
12. Dr. Richard Osgood, Army Natick Soldier Research, Development, and Engineering Center.

13. Dr. Ashok Sood, Magnolia Optical Technologies Inc.
14. NanoSafety Laboratories, Inc; nanotechnology applications related to prediction, detection, and measurement of environmentally stimulated toxicological cellular responses; board advisor and collaborator (Yeh).
15. NanoDynamics Life Sciences, Inc.; development of nanomaterials and applications to life sciences; collaborator (Yeh).

Invited lectures, S.O. Kelley, "Ultrasensitive Biomolecular Detection using DNA-Modified Nanostructures," delivered at
16. Auburn University, November 2004
17. Florida Atlantic University, December 2004
18. University of Maryland, Baltimore, February 2005
19. Facultés Universitaires Notre-Dame de la Paix, February 2005
20. Georgia Institute of Technology, February 2005
21. University of Toronto, March 2005
22. University of Utah, March 2005
23. University of North Carolina, April 2005
24. Penn State University, April 2005
25. University of California, San Francisco, May 2005
Invited lectures, S.O. Kelley, "Interfacing and Templating Nanostructures with DNA," delivered at
26. Massachusetts Institute of Technology, September 2005
27. Columbia University, October 2005
28. University of California, San Diego, October 2005
29. University of Vermont, October 2005
30. California Institute of Technology, *October 2005*
31. *University of California, San Diego*, October 2005
32. *Colorado State University*, October 2005
33. *University of California, Berkeley*, October 2005
34. *University of Minnesota*, November 2005
35. *Carleton College*, November 2005
36. *Brown University*, December 2005
37. *University of Illinois*, December 2005
38. *University of Wisconsin*, December 2005
39. *University of Washington*, December 2005, "Exploring Cellular Processes with Peptide-Based Probes and Nanoscale Sensors"
40. S.O. Kelley, development of electrical detection systems with **GenoRx, Inc.**
41. S.O. Kelley, evaluating the practicality of using nanowire electrodes for pharmacogenetics with **Xanthus Pharmaceuticals**
42. Contarino, Mark R.; Kamat, Vishal; Keough, Edward; Babu, Nadarajan S.; Sergi, Mauro; Ishino, Tetsuya; Papazoglou, Elisabeth; Chaiken, Irwin M.; Tyagi, Som; Pourrezaei, Kambiz. "Bio-nano-optics for Native Cellular Investigations", in SPIE Optics East, August 25-28, Philadelphia, PA USA (2004)
43. EIC Labs, Norwood, MA. Contact is Dr. David Rauh. drauh@eiclabs.com. Array-based nanotube-based biosensors (Martin)
44. The Broadley James Company, Irvine, CA. Contact is Mr. Scott Broadley. sbroadley@broadleyjames.com. Nanofluidic reference electrode technology (Martin)

Invited lectures, S. Zhang, delivered at
45. University of Tokyo, March, 2005
46. Institute of Biophysics, Chinese Academy of Science, Beijing, China, March 2005
47. Intel Corp. San Jose, April 2005
48. The Schrödinger Society, Vienna, Austria, April 2005
49. Tufts University Medical School, Boston, April 2005
50. Institute of Chemistry, Chinese Academy of Science, Beijing, China, August 2005
51. Vanderbilt University, Nashville, TN, Oct. 2005.

52. R. Beresford submitted a DARPA proposal for a spin-off of MURI technology (virus detection chip) with co-PI S. Kelley and industry partner W. Valdivia (Orion Biosciences).
53. J.I. Yeh, "Integration of High-Resolution Biomolecular Structures and Function in Nanomedicine and Nanosensor Applications" (November 1, 2005). Speaker, International Congress of Nanotechnology, San Francisco, California.
54. J.I. Yeh, "Integration of High-Resolution Biomolecular Structures and Function in Nanomedicine and Nanosensor Applications" (October 06, 2005). Speaker, Science 2005, University of Pittsburgh.
55. J.I. Yeh, "Oxidative Sensing and Glycerol Metabolism: from crystal structures to nanobiotechnology applications". (May 20, 2005). Speaker, UCLA, Department of Biochemistry Dept. Seminar, Los Angeles, California.
56. J.I. Yeh, "Improvement of Crystal Quality by Time-Controlled Annealing" (August 24, 2005). International Union of Crystallography, Florence, Italy.
57. J.I. Yeh, "Structures, Functions, and Applications of Enzymes Involved in Oxidation and Glycerol Metabolism" (April 04, 2005). Department of Chemistry and Biochemistry, University of Delaware, Wilmington, DE.
58. J.I. Yeh, "Application of Three-Dimensional Structures to Nanobiotechnology Application" (March 23, 2005). Speaker, University of West Virginia, Nanotechnology Seminar Series, Morgantown, Virginia.
59. J.I. Yeh, "Manual Nanocrystallization and Enhancing Diffraction *in-situ* by Annealing" (March 18, 2005). SER-CAT Symposium, St. Jude Research Hospital, Memphis, Tennessee.
60. J.I. Yeh, "Nanowiring of a Redox Enzyme by a Metallized Peptide" (March 02, 2005). Speaker, UCLA, Department of Bioengineering Dept. Seminar, Los Angeles, California.
61. J.I. Yeh, "Structural and Functional Studies of Proteins Involved in Oxidative and Glycerol Metabolism". (February 15, 2005). Speaker, University of Maryland, Department of Pharmacology Seminar Series, Baltimore, Maryland.
62. J.I. Yeh, "NADH Specific Signal Generation through an Electrochemically Active Biocatalytic Assembly" (January 21, 2005). Speaker. UCLA, CMISE Seminar, Los Angeles, California.
63. J.I. Yeh, "Nanoscale Methods for Protein Crystallization" (January 18, 2005). Speaker. Protein Crystallography in Drug Discovery Conference, San Francisco, California.
64. J.I. Yeh, "NADH Specific Signal Generation through an Electrochemically Active Biocatalytic Assembly" (November 10, 2004). Speaker. International Congress of Nanotechnology, San Francisco, California.
65. J.M. Xu, "Nanoelectronics – evolution from CMOS to Nanotube, DNA and Protein", Kyungprook National University, Dae-Gu, August 12, 2003.

66. J.M. Xu, "Nanoelectronics – evolution from CMOS to Nanotubes and DNAs", **Plenary Speech**, 11th Canadian Semiconductor Technology Conference, Ottawa, August 18, 2003.

67. J.M. Xu, "Nanoelectronics and BioMed Engineering", BioMed Engineering Program, Seminar Series, Brown Sept. 25, 2003

68. J.M. Xu, "Carbon Nanotube as Sensor Materials", 2004 Symposium of Nanomaterials for Defense Applications, Maui, Hawaii, Feb 18-24, 2004

69. J.M. Xu, "Nanoelectronics – Evolution from CMOS, to Nanotube, DNA, Protein", **Keynote**, Nagoya University 1ST Symposium on Micro, Nano, Electro-Mechanical Systems, Nagoya, Japan, March 9, 2004

70. J.M. Xu, M. Tzolov, M. Jouzi, "Carbon Nanotube Electro-Mechanical Coupling", 2004 GOMAC, Monterey, CA, March 14-19, 2004

71. J.M. Xu, "Carbon Nanotube Quantum Electro-Mechanical Couplings", Boston College Workshop on Meso and Nano Materials, March 19-20, 2004

72. J.M. Xu, "Carbon Nanotube Sensory", The 3rd US-Korea Workshop on Nanostructured Materials and Nanomanufacturing, Seoul, May 10-11, 2004

73. J.M. Xu, "Nanoelectronics – Evolution from CMOS to Nanotube, DNA and Protein", The 1st US-Korea Workshop on Nanoelectronics, Seoul, May 10-11, 2004

74. J.M. Xu, "Interfacing Biomolecules and Nanoelectronics", Gordon Conference on Computational Chemistry, Plymouth, NH, July 4-9, 2004

75. J.M. Xu, "Carbon Nanotubes as Sensor Materials", Colloquium of Carbon Nanoforms, Carbon 2004, Providence, RI, July 11, 2004

76. Z. Xia, B. Sheldon, W. Curtin, H. Li, J. Liang, B. Chang, J.M. Xu, **(Keynote Lecture)** "Toughening Mechanisms in Nanotube Reinforced Ceramic Composites", Carbon 2004, July 15, Providence, RI, 2004

77. J.M. Xu, "Quantum Electro-Mechanical Couplings of Carbon Nanotubes", Nano Korea 2004, Seoul, Korea, August 24-26, 2004

78. J.M. Xu, "Interfacing Biomolecules and Nanoelectronics", Seoul National University, August 26, 2004

79. J.M. Xu, "Nano-Bio-technology Fusion", Brown Enterprises Forum, Sept 23, 2004.

80. J.M. Xu, ""A big deal about little things - interfacing biomolecules with nanoelectronics", Brown Home-Coming and Parent Days Faculty Forum, Oct 23, 2004

81. J.M. Xu, "Making a big deal out of little things – At the interface between nanoelectronics and biolmolecules", Institute For Defense Analyses, Washington D.C., Dec 17, 2004

82. J.M. Xu, "Growth and Applications of Highly Ordered Arrays of Nanoposts" 1st International Conference on One-Dimensional Nanostructures, Taipei, Jan, 9-15, 2004

83. J.M. Xu, "Explorations of Periodic Nanometric Superstructures in Photonics and Bionanoelectronics", Dept. of EECS Seminar, University of Michigan, Feb. 15, 2005.

84. J.M. Xu, "Nanoneedles for bio-molecular interfacing and intracellular delivery and probing", Marine Biology Laboratory, Woods Hole, MA, April 27, 2005

85. J.M. Xu, "Carbon Nanotube Arrays and Potential Applications in Lightweight Functional Composites and Solarless Solar-Cells", Textron Systems, Boston, May 18, 2005

86. J.M. Xu, "Nanotube Electronics: An enabling platform for beyond-CMOS technologies", IEEE EDS Virginia/DC chapter meeting and Gorge Mason University, June 28, 2005.

87. J.M. Xu, "DNA – Nanotube Conjugation for Bio-electronics and Nano-electronics", Nano-structurre Science & Technology Symposium 2 (NSTS-2) at UKC2005 (US-Korea Conference on Science, Technology, & Entrepreneurship), UC Irvine, August 11-13.

88. J.M. Xu, "Nanotube Electronics – an enabling platform for beyond-CMOS explorations", Oak Ridge National Lab, August 22, 2005

89. J.M. Xu, " Biomolecular – Carbon Nanotube electronics", Workshop on Silicon-based Nanodevices (SINANO), September 16th, 2005, Grenoble, France.

90. C.R. Martin, "Nanoscience in Bioanalytical Chemistry," University of Michigan, Ann Arbor, MI, April 18, 2005.

91. C.R. Martin, "Bioconjugated Nanotubes for Biosensing and Bioseparations," Biomedical Engineering Roundtable, U.S. National Academies, Washington, DC, October 1, 2004.

92. C.R. Martin, "Frontiers in Chemical Research," Texas A&M University, November 1-3, 2004, "Template Synthesis of Nanomaterials."

93. C.R. Martin, "Biofunctionalized Nanotubes for Bioseparations and Biosensing," Cornell University, Ithaca, NY October 7, 2004.

94. C.R. Martin, "DNA Biofunctionalized Nanotube Membranes," Georgia Institute of Technology, Atlanta, GA, May 20, 2004.

95. C.R. Martin, "DNA Biofunctionalized Nanotube Membranes," Department of Materials Science and Engineering, University of Florida, Gainesville, FL, March 30, 2004.

96. C.R. Martin, "Nanotube Membranes and the Bio/Nano Interface," Massachusetts Institute of Technology, Cambridge, MA February 12, 2004.

97. C.R. Martin, "Nanoscience in Bioanalytical Chemistry," Florida Section ACS Meeting, Orlando, FL, May 6, 2005.

98. C.R. Martin, "Biosensing with Nanotube Membranes," Pittcon 2005, Orlando, FL, March 1, 2005.

99. C.R. Martin, "Nanotube-Based Artificial Voltage-Gated Ion Channels," Pittcon 2005, Orlando, FL, March 2, 2005.

100. C.R. Martin, "Electrochemistry of Nanotube-Based Ion-Channel Mimics," Gordon Conference on Electrochemistry, February 12, 2005.

101. C.R. Martin, "DNA and Protein Transport in Synthetic Nanopore Membranes," American Physical Society Meeting, Montreal, March 23, 2004.

102. C.R. Martin, "Moving Biomolecules Through Nanotubes," PitCon 2004, Chicago, IL March 8, 2004.

103. C.R. Martin, "Short Course on Nanotechnology," University of Venice, Venice Italy, July 12 and 13, 2004.

104. C.R. Martin, "Nanoscience in Bioanalytical Chemistry," HRL, Malibu, CA, July 18, 2005.

105. C.R. Martin, "Nanoscience in Bioanalytical Chemistry," University of California at Irvine, February 7, 2006.

106. C.R. Martin, "Nanotube and Nanopore Biosensors," Pittcon 2006, Orlando, FL, March 13, 2006.

107. C.R. Martin, "Nanoscience in Bioanalytical Chemistry," HRL, Malibu, CA, July 18, 2005.

108. C.R. Martin, "Nanoscience in Bioanalytical Chemistry," Plenary lecture at the 11th International Conference on Electroanalytical Chemistry, Bordeaux, France, June 12, 2006.

109. J.I. Yeh, "Membrane Protein Crystallization" (August 28-Sept.4, 2006). Lecturer and Instructor, EMBL-Hamburg, Practical Course on Protein Expression, Purification, and Crystallization, Hamburg, Germany.

110. J.I. Yeh, "Calorimetric Approaches to Characterizing Effects of Additives on Protein Crystallization" (August 16-20, 2006). Speaker. International Conference on Crystallization of Biological Macromolecules. Quebec City, Canada.

111. J.l. Yeh, "Structural and Functional Studies of Enzymes Involved in Oxidative and Glycerol Metabolism" (June 1-3, 2006). Speaker. 36th Mid-Atlantic Macromolecular Crystallography Meeting.

112. J.l. Yeh, "Integration of High-Resolution Biomolecular Structures and Function in Nanomedicine and Nanosensor Applications" (Feb 16-17, 2006). Speaker. University of Florida, Department of Chemistry, Center for Bio-Nano Interface, Gainesville, Florida.

113. J.l. Yeh, "Ultra-sensitive Nanodetection Methods Based on Biomolecules" (February 3, 2006). Speaker, Nanotoxicology Meeting, California Nano Sciences Institute, UCLA.

114. J.l. Yeh, "Structural and Functional Studies of Enzymes Involved in Oxidative and Glycerol Metabolism" (February 2, 2006). Speaker, Department of Medicine, UCLA.

115. J.l. Yeh, "Membrane Protein Structural Studies and Applications" (December 13, 2005). Speaker, Sandia National Laboratory, Albuquerque, New Mexico.

116. R. Beresford, Microfluidic Nanoelectrode Cell Array for Direct Electrochemical Detection of Biomolecules, Showcase of Nanomedicine, Brown University, Providence, Rl, June 2006

117. R. Beresford, Session Chair, Gordon Conference on MEMS Technology and Biomedical Applications, New London, CT, June 2006.

118. R. Beresford, "Light Ideas," Brown University, Sarah Doyle Women's Center "Cliff Notes" Series, March 2007.

119. Nikolai Lebedev: Light Energy Conversion by Photosynthetic Proteins at Inorganic Electrodes. – Bio-inspired Chemistry for Energy, NSF strategic group meeting, Washington, DC, May 14-15, 2007

120. S.O. Kelley, "Nanostructures for Ultrasensitive Electrochemical Biosensing," *Discovery 2 Diagnostics: Chips to Hits*, Boston, MA, Sept. 2006.

121. S.O. Kelley, "Ultrasensitive Biosensing Using Nanowire Electrodes," *University of Rochester*, Sept. 2006.

122. S.O. Kelley, Electroactive Nanowires For Medical Diagnostics," *Canadian Undergraduate Technology Conference*, Toronto, Ontario, Jan. 2007.

123. S.O. Kelley, "Nanoscale Electrodes for Medical Diagnostics" University of Waterloo, *April 2007.*

124. Jimmy Xu, "Nanotube electronics – beyond CMOS scaling", panel presentation, IEEE Nano Materials, Devices, and Circuits Meeting, Korea, Oct. 21-26, 2006

125. Jimmy Xu, "Carbon Nanotubes for Intracellular Delivery and Molecular Detection", Department of Orthopedics, Brown Medical School, "Frontier of Musculoskeletal Research" seminar series, Feb 28, 2007

126. Jimmy Xu, "Highly Ordered Nanotube Array approach to carbon-based RF electronics", DARPA Workshop on "Carbon Electronics for RF Applications", Washington DC, March 19-20, 2007

127. Jimmy Xu, "Silicon Lasers - an anti-physics wish or a real possibility?", Colloquium, Physics Department, Northeastern University, Oct 11, 2007.

128. Jimmy Xu, "Information Technology - Pending Crisis and Great Opportunity", Faculty of Engineering, National University of Singapore, Jan 22, 2008 .

129. Jimmy Xu, "In pursuit of silicon laser", Department of Materials Science and Engineering, National University of Singapore, Jan 22, 2008.

130. Jimmy Xu, "Formation and infrared sensing of highly ordered carbon nanotube - silicon heterojunction arrays", June 12, 2008, Army Natick Soldier Research Development and Engineering Center, Natick, MA.

131. Irwin Chaiken, *Protein interactions on and in cells,* Affinity 2007, New York City: July 2007.
132. Irwin Chaiken, *Parallel versus anti-parallel alignment in fluorescently labeled peptide coil,* REU Sensors Day, Drexel, August 2007.
133. Irwin Chaiken, participated in plan with Ben Franklin Technology Partners and Prime Synthesis Inc. to develop a glass-based analytical method to examine interaction patterns in cells using coiled coil modular self-assembling system.
134. J.I. Yeh, "Oxidative Sensing and Glycerol Metabolism in Pathogenic Bacteria: from Structures to function" (April 23, 2007). Paul Scherr Institute, Swiss Light Source, Zurich, Switzerland,
135. J.I. Yeh, "Coordinated Nanobiosensors: Integration of 3D structures with Nanoelectrode Platform." (March 05, 2007). Speaker. University of Pittsburgh McGowan Institute Retreat, Pittsburgh, PA.
136. J.I. Yeh, NanoSafety Laboratories, Inc; nanotechnology applications related to prediction, detection, and measurement of environmentally stimulated toxicological cellular responses; board advisor and collaborator.
137. "Nanoscience in Bioanalytical Chemistry, 1, Fabrication and Biofunctionalization of Nanotube Membranes," University of Pittsburgh, Pittsburgh Conference Lecture, May 7, 2006.
138. "Nanoscience in Bioanalytical Chemistry, 2, Sensing and Single-Molecule Counting with Nanotube Sensors," University of Pittsburgh, Pittsburgh Conference Lecture, May 7, 2006.
139. C.R. Martin, NIH STTR and a DARPA SBIR with EIC Labs, Norwood, MA. The PI is Dr. David Rauh of EIC. These grants deal with the biofunctionalized conical gold nanotubes that are the focus of the Martin MURI effort.
140. S.O. Kelley, "Nanoscale Electrode Platforms for Ultrasensitive Biosensing," *Pittcon,* Chicago, IL, Feb, 2008.
141. S.O. Kelley, "Nanoscale Electrodes for Medical Diagnostics" University of British Columbia, *April 2008.*
142. S.O. Kelley, "Nanoscale Electrodes for Medical Diagnostics," University of Victoria, *April 2008*
143. S.O. Kelley, "Nanoscale Electrodes for Medical Diagnostics," Simon Fraser University, *April 2008*
144. S.O. Kelley, "Nanoscale Electrodes for Medical Diagnostics," University of Pittsburgh, *April 2008.*
145. J.I. Yeh, "Structure of Glycerol-3-Phosphate Dehydrogenase, an Essential Integral Membrane Enzyme Involved in Modulation of Respiration, Energy Status, and Phospholipid Biosynthesis". (September 03-05, 2009). Invited Speaker. Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan.
146. J.I. Yeh, "Ultra High Resolution Structures of Peptide Nucleic Acids: Conformations and Applications". (October 23, 2008). Seminar Speaker. Carnegie Mellon University, Department of Chemistry, Pittsburgh, PA.
147. J.I. Yeh, "Diffraction and Symmetry in Macromolecular Crystallography". (October 17, 2008). Invited Speaker. Course on Biological Crystallography, Stanford Linear Accelerator Center. Stanford University, Palo Alto, CA.
148. J.I. Yeh, "Structures and Functions of Membrane and Soluble Enzymes Involved in Glycerol Metabolism: from Regulatory Mechanisms to Modulation of Respiration and

Phospholipid Biosynthesis" (September 10-11, 2008). CNRS-ParisTech, Thiverval-Grignon, France.

149. J.I. Yeh, "Membrane Protein Crystallization" (September 1-9, 2008). Lecturer and Instructor, EMBL-Hamburg, Practical Course on Protein Expression, Purification, and Crystallization, Hamburg, Germany.

150. J.I. Yeh, "Structure of Glycerol-3-Phosphate Dehydrogenase, an Essential Integral Membrane Enzyme Involved in Modulation of Respiration, Energy Status, and Phospholipid Biosynthesis" (March 26,27, 2008). Seminar Speaker. Case Western University, Department of Biochemistry, Cleveland, Ohio.

151. J.I. Yeh, "Structure of Glycerol-3-Phosphate Dehydrogenase, an Essential Integral Membrane Enzyme Involved in Modulation of Respiration, Energy Status, and Phospholipid Biosynthesis" (March 11, 2008). Seminar Speaker. Wake Forest University, Department of Biochemistry, Greensboro, NC.

4. Patents 13

1. Kelley et. al., "Electrocatalytic DNA Detection", PCT application filed 5/04.
2. N. Kouklin and J.M. Xu, "Controlled Assembly of Molecular-Scale Carbon Nanotube Probes", filed 2/04.
3. J. Yeh, et. al., Biomolecular Conduits of Electronic Signals (in preparation)
4. Zhang, S., Kiley, P., Zhao, X & Baldo, M. Peptide surfactants stabilize functional membrane proteins and uses thereof (MIT Case. 10618, To be filed, 2004).
5. S.O. Kelley et al., Electrocatalytic Nucleic Acid Hybridization Detection, Filed 4/12/05, U.S.S.N. 60/670,406
6. S.O. Kelley et al., Electrocatalytic Protein Detection, Filed 4/12/05, U.S.S.N. 60/670,406
7. M. Contarino and I. Chaiken. Patent application first stage - Drexel Docket #05-0621: Direct nanoscale conversion of fluorescence to electronic event; a novel biosensor to monitor protein assembly in native environments
8. C.R. Martin et al., "Nanoparticle delivery system," UPSTO Serial # 10/274,829, filed October 21, 2002, patent pending.
9. C.R. Martin et al., "Detoxification and decontamination using nanotechnology therapy," USPTO Serial # 09/978344, filed October 16, 2001, patent awarded.
10. W. Kim and J.M. Xu, "Massively Multiplexed Fluorescence In Situ Hybridization", filed 7/05.
11. S.O. Kelley et al., Electrocatalytic Nucleic Acid Hybridization Detection, Filed 4/12/05, U.S.S.N. 60/670,406
12. S.O. Kelley et al., Electrocatalytic Protein Detection, Filed 4/12/05, U.S.S.N. 60/670,406
13. J.I. Yeh, Provisional filing on PS Biosensor

5. Impact on teaching 11
(new courses, or new content in existing courses)

1. Kelley runs a literature workshop over the last two years for graduate students focused on advances in the uses of nanomaterials for biosensing.

2. New research content incorporated in Drexel courses in Biomedical Engineering, *Exploring the Protein Interactome, Receptor Machines*, Information Sciences *Introduction to Proteins and Opportunities for Data Mining and Bioinformatics*, Bioscience and Biotechnology: *Recognition, Antagonism and Affinity Science; Lessons from Studies of Receptor Machines*, and Material Science and Engineering: *Recognition, Antagonism and Affinity Science; Lessons from Studies of Receptor Machines*
3. A new course "Computational biology" was initiated this year at the departments of Mathematics and Computer Sciences of George Mason University
4. Further strengthened and enhaced the MIT Bioengineering combined undergraduate and graduate course, BE342/442, *"Molecular structures of Biological Materials: Structure, Function and Self-assembly"*
5. New Course Developed Spring 2005   BI194/PH199 Molecular Biophysics (JIY Organizing Instructor): course taught theory and application of techniques to characterization of biomolecules.
6. A new course has been introduced at the graduate student level that incorporates nano and bio electronics, EN263 "Electrical and Optical Properties of Materials and Biomolecules" (J.M. Xu)
7. Molecular Biophysics Data and Literature Club: JIY developed new course for graduate students and postdocs, focused on scientific data analysis and new literature in areas related to structural biology, molecular biophysics, and nanobiotechnology.
8. Fundamentals of Molecular Biophysics: JIY co-organizer of core course for graduate students, focused on teaching fundamental principles and methodologies related to structural biology molecular biophysics, and biochemistry. Additional theory and techniques related to nanobiotechnology added this year.
9. New special topics course at the graduate level, EN292-S29 "Nanoelectronics" was developed and taught in spring 2006 (R. Beresford)
10. New contents in Protein Interactions lectures to graduate students in Bioscience and Biotechnology Proteins Course and in Biomedical Graduate Group Core Biochemistry Course (I. Chaiken).
11. New contents in Biosensors classes for Biochemistry/Molecular Biology Ph.D. students (I. Chaiken).

6. Awards **25**

1. Sloan Research Fellowship to Kelley; Kelley named as Technology Review's "Top 100 innovator".
2. D. I. Ionata Award for the most creative thesis to Karl Hanson (Xu group).
3. Dreyfus Teacher-Scholar Award (Kelley).
4. BC Distinguished Research Award (Kelley).
5. Pittsburgh Conference Achievement Award 2006 (Kelley).
6. GAANN Fellowship, NSF IGERT Fellowship to M. Contarino (Chaiken group).
7. ISI's Highly Cited Author Ranking in Materials Science, 2005. http://isihighlycited.com/ (Martin).
8. Fellow of the Electrochemical Society, 2005 (Martin).

9. University of Florida Research Foundation Professorship, 2005-2007 (Martin).
10. Florida Award of the Florida Section of the American Chemical Society, May 5, 2005. (Martin).
11. Colonel Allan R. and Margaret G. Crow Professorship in Chemistry, 2004 (Martin).
12. March of Dimes Jonas Salk Health Leadership Award to Yeh (09/04).
13. Finalist of the 2005 Saatchi & Saatchi Award for World Changing Ideas (Zhang).
14. Fellow of the Institute of Physics (UK), 2004 (Xu).
15. Charles C. Tillinghast University Professor, Brown University, 2005 (Xu).
16. Guggenheim Fellow, 2005, (Xu).
17. Named University Distinguished Professor July 2006 (Martin).
18. Named U.S. Senior Editor of the journal *Nanomedicine* April 2006 (Martin).
19. Inaugural Best Ph.D Thesis in Engineering Award, 2006, Brown University (Sylvain Cloutier, Xu graduate student).
20. Sigma Xi Prize, 2006, Brown University (S. Cloutier, Xu group).
21. Charles R. Martin won a Nano 50 Award from Nanotech Briefs, June 2007.
22. Jimmy Xu, finalist of the Thatcher H. Smith "Creativity in Motion" Prize.
23. Alan Berman Research Publication award of 2008 (Lebedev).
24. C.R. Martin will be awarded the Charles N. Reilley Award of the Society for Electroanalytical Chemistry, to be presented March 2009.
25. C.R. Martin won the Senior Faculty Research Award, University of Florida Chapter of Sigma Xi, March 2008.